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TITLE: **Melanocortin and Opioid Peptide Interactions in the Modulation of Binge Alcohol Drinking**

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14. ABSTRACT Frequent binge drinking is associated with numerous negative short- and long-term consequences, including an increased risk of accidental injury, violent behavior, depression, heart disease, and type 2 diabetes. While illicit drug use and cigarette smoking both decreased significantly in the US military between the period of 1980 to 2002, heavy alcohol use increased. In fact, heavy alcohol use and binge drinking are observed in 27% of the military population. Identifying neurochemical pathways in the brain that modulate binge drinking may provide insight into pharmaceutical treatments that could protect against this dangerous behavior. Recently identified candidates for modulating binge drinking are the melanocortin (MC) peptides, such as α-melanocyte stimulating hormone (α-MSH), and the opioid peptide β-endorphin which are produced in the same brain neurons. The specific aims proposed below will test the guiding hypothesis that stimulation of MC receptor and blockade of opioid receptor protect against excessive binge-like alcohol drinking and intoxicating blood alcohol levels (BALs) in an animal model of binge drinking. The aims will also determine if MC receptor (MCR) agonists and opioid receptor antagonists interact to protect against binge-like alcohol drinking in a synergistic manner.

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INTRODUCTION: Alcohol (ethanol) dependence and relapse in abstinent alcoholics are major health problems in the United States and neurochemical pathways that modulate these disorders are currently under investigation. However, heavy alcohol use and binge alcohol drinking have received far less attention. A 'binge' is defined by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) as a pattern of drinking that produces blood alcohol levels (BALs) greater than 0.08% (NIAAA, 2004). The pattern of alcohol drinking required to produce these BALs is about 5 and 4 drinks in 2-hours for the average adult male and female, respectively. As with all patterns of alcohol abuse, frequent binge drinking is associated with numerous negative short- and long-term consequences. Binge drinking increases the risk of accidental injury (Gmel et al., 2006), increases mood disorders (Okoro et al., 2004), increases aggressive and violent behavior (Shepherd et al., 2006), and impairs decision making and judgment (Goudriaan et al., 2007). Furthermore, heavy binge drinking has been linked to long-term health consequences including heart disease, high blood pressure, and type 2 diabetes (Fan et al., 2008). Perhaps most alarming is the finding of increased risk for developing alcohol dependence in individuals that binge drink early in life (Hingson et al., 2005; Hingson et al., 2006; Miller et al., 2007). Importantly, while illicit drug use and cigarette smoking both decreased significantly in the US military between the period of 1980 to 2002, binge drinking and heavy alcohol use increased and occurs in 27% of the military population (Bray et al., 2002). Given that the rate of binge drinking in the civilian population is about 15% (SAMHSA, 2003), individuals in the military are at an increased risk of regular binge drinking and thus all the health risks associated with this disorder. *Identifying neurochemical pathways in the brain that modulate binge drinking may provide insight into novel pharmaceutical treatments that could protect against this dangerous behavior.* Recently identified candidates for modulating binge drinking are the melanocortin (MC) peptides, such as α-melanocyte stimulating hormone (α-MSH), and the opioid peptide β-endorphin which are both cleaved from the polypeptide precursor proopiomelanocortin (POMC). The specific aims of this grant tested the guiding hypothesis that MC receptor signaling protects against excessive binge-like alcohol drinking and intoxicating blood ethanol concentrations (BECs) in an animal model of binge drinking. The aims also determine if MC receptor (MCR) agonists and opioid receptor antagonists interact to protect against binge-like alcohol drinking in C57BL/6J mice in a supraadditive (synergistic) manner. **Specific Aims: Specific Aim 1** will test the hypothesis that binge-like alcohol drinking was associated with a significant reduction of α-MSH levels in candidate brain regions of C57BL/6J mice, and if this reduction of α-MSH will become more robust following repeated binge episodes. This aim also determined if repeated binge episodes promote increases of subsequent ethanol drinking in mice. **Specific Aim 2** tested the hypothesis that central infusion of MCR agonist protected against, and MCR antagonist augmented, binge-like alcohol drinking in C57BL/6J mice via the MC-3 receptor (MC3R) or MC4R. Mutant mice lacking MC3R or MC4R were used to determine the receptor(s) that are involved. **Specific Aim 3** tested the hypothesis MCR agonists and opioid receptor antagonists interact to protect against binge-like alcohol drinking in C57BL/6J mice in a supraadditive (synergistic) manner.

BODY (NOTE: Experiments described below are organized based on the experimental tasks that were proposed in this grant):

Tasks 1 : Will determine if binge-like alcohol drinking will be associated with a significant reduction of α-MSH levels in candidate brain regions of C57BL/6J mice, and if repeated binge episodes will be associated with a progressive increase of binge-like drinking. Task 1 entailed two goals, to determine if a history of binge-like ethanol drinking in mice would be associated with reduced α-MSH immunoreactivity (IR) in candidate brain regions, and if a history of binge-like ethanol drinking would promote a subsequent increase of ethanol drinking,

consistent with a dependence-like state. *For the first goal*, separate groups of mice experienced 1, 3, or 6 cycles (1-EtOH, 3-EtOH, and 6-EtOH) of binge-like ethanol drinking using the "drinking in the dark" (DID) protocol, a model of binge-like ethanol drinking in C57BL/6J mice. On Days 1-3, water bottles were removed from the home cage 3 h into the dark cycle. Bottles containing either 20% (v/v) ethanol or 3% (w/v) sucrose (a control for the specificity of the effects of ethanol on α-MSH IR) were weighed to the nearest 0.1 gram and immediately placed on cages. After 2 h the bottles were removed from the cages and again weighed to the nearest 0.1 g. Following this 2 h period the original water bottles were replaced on the cages and the mice left undisturbed until the next day. On Day 4, an identical procedure was followed with the exception that ethanol (or sucrose) access was extended to 4 h. Control ethanol and sucrose bottles were placed on an empty cage every day of the study to control for fluid spillage and evaporation. The average fluid lost from these control bottles was determined at the end of each binge-like drinking session and the daily ethanol and sucrose consumption values were adjusted accordingly. Following the final drinking session, all animals were sacrificed and the expression of α-MSH and agouti-related protein (AgRP; an endogenous MCR antagonist) in key brain regions was examined using immunohistochemical techniques.

Mice in the 1-EtOH, 3-EtOH, and 6-EtOH groups (n = 10/group) consumed similar amounts of ethanol during the final binge-like drinking session (5.71 ± 0.50, 5.77 ± 0.35, 6.36 ± 0.33 g/kg/4 h, respectively; $F(2,27)= 0.811$, $p=0.455$), as seen in **Figure 1A**. Similarly, there were no significant differences between groups (n = 9/group; 3 blood samples lost before analysis) in terms of BECs achieved during this session (133.06 ± 17.84, 162.59 ± 9.64, and 188.82 ± 19.44 mg/dl; $F(2,24)=2.958$, $p=0.071$; **Figure 1B**). Thus despite experiencing different numbers of binge-like ethanol drinking cycles, mice in all ethanol-drinking groups (1-EtOH, 3-EtOH, 6-EtOH) displayed similar ethanol consumption and BECs on the final binge-like drinking session. Additionally, mice in the 1-Suc, 3-Suc, and 6-Suc groups (n = 10/group) did not significantly differ in terms of total sucrose consumed during the final binge-like drinking session (246.52 ± 29.5, 272.90 ± 35.08, and 350 ± 28.16 ml/kg/4h, respectively; $F(2,27)=3.005$, $p=0.066$; **Figure C**). Finally, animals in the ethanol-drinking groups (26.45 ± 0.36, 27.17 ± 0.32, and 26.98 ± 0.49 g) and sucrose-drinking groups ((26.82 ±0.40, 28.33 ± 1.05, and 27.44 ± 0.37 g) displayed similar body weights at the end of the study ($F(5,54)=1.332$, $p=0.265$). Representative photomicrographs of α-MSH IR from ethanol-binging mice (top row) and water-drinking control mice (bottom row) can be seen in **Figure 2**. Images are from the lateral hypothalamus (LH; A and E), dorsomedial hypothalamus (DMH; B and F), arcuate nucleus of the hypothalamus (ARC; C and G), and the paraventricular nucleus of the hypothalamus (PVN; D and H).

LH DATA: Figures 3A-B show α-MSH IR in the LH following 1, 3, or 6 binge-like drinking sessions of 20% ethanol or 3% sucrose. Binge-like ethanol drinking was associated with a significant downregulation of α-MSH IR ($F(3,27)=5.481$, $p=0.004$); post-hoc analysis confirmed that mice in all binge-like ethanol drinking groups (1-EtOH, 3-EtOH, and 6-EtOH) showed blunted α-MSH IR relative to water drinking control animals. Importantly, this decrease appeared after just one binge-like drinking cycle, suggesting that α-MSH IR within this region is particularly sensitive to the effects of binge-like ethanol drinking. A non-significant one-way ANOVA ($F(3,28)=0.785$, $p=0.512$) performed on data from animals that experienced 1, 3, or 6 cycles of sucrose intake (1-Suc, 3-Suc, 6-Suc) confirmed that this decrease in α-MSH IR was specifically associated with binge-like ethanol consumption.

DMH DATA: α-MSH IR following 1, 3, or 6 binge-like drinking sessions of 20% ethanol or 3% sucrose is shown in **Figures 3C-D**. Relative to control mice, mice that experienced multiple binge-like ethanol drinking sessions showed a significant reduction in α-MSH IR ($F(3,27)=3.561$, $p=0.027$). Post-hoc analysis confirmed that mice in both the 3-EtOH and 6-EtOH groups showed a significant reduction in α-MSH IR relative to WAT mice. A similar analysis performed in sucrose binging groups showed no differences between sucrose drinking groups and the WAT group ($F(3,22)=0.165$, $p=0.919$). Thus the reduction in α-MSH IR in the DMH was specific to binge-like ethanol consumption and only appeared following 3 or 6 binge-like ethanol drinking cycles.

ARC DATA: We assessed α-MSH IR in the ARC following 1, 3, or 6 binge-like drinking sessions of 20% ethanol or 3% sucrose (**Figures 3E-F**). A one-way ANOVA revealed that repeated ethanol exposure also blunted α-MSH IR in this region ($F(3,24)=3.258$, $p=0.039$) and post-hoc analysis confirmed that α-MSH IR in the 6-EtOH group was blunted relative to both WAT and 1-EtOH groups. A non-significant ANOVA on data from the sucrose drinking groups confirmed this decrease in α-MSH IR was specific to multiple cycles of binge-like ethanol consumption ($F(3,25)=0.736$, $p=0.540$).

PVN DATA: Figures 3G-H show α-MSH IR in the PVN following repeated binge-like drinking cycles of 20% ethanol or 3% sucrose. A one-way ANOVA performed on the ethanol drinking groups was significant ($F(3,30)=3.109$, $p=0.041$), and post-hoc analysis revealed that only the 6-EtOH group had significantly reduced staining relative to the WAT control group. A similar analysis confirmed that there were no significant changes in α-MSH IR in sucrose drinking groups ($F(3,28)=1.906$, $p=0.152$). Thus, the decrease in α-MSH IR was specific to ethanol binge-like consumption and required multiple binge-like drinking cycles to emerge.

AGRP DATA: Given previous data from our laboratory showing that elimination of endogenous AgRP protects against binge-like ethanol consumption (Navarro et al., 2009), we examined central AgRP IR following 1, 3, or 6 binge-like drinking cycles with either 20% ethanol or 3% sucrose. Representative photomicrographs of AgRP IR in the PVN of ethanol binge-like drinking mice (A) and water drinking control mice (C) can be seen in **Figure 4**. AgRP IR in the PVN following 1, 3, or 6 binge-like drinking sessions of 20% ethanol or 3% sucrose is shown in **Figure 5A-B**. Binge-like ethanol drinking was associated with an increase in AgRP IR in this region ($F(3,26)=19.842$, $p<0.001$); post hoc analysis confirmed that the 6-EtOH group showed significantly increased AgRP IR relative to WAT, 1-EtOH, and 3-EtOH groups. This increase was specific to ethanol, as a similar analysis performed on the sucrose drinking groups was not significant ($F(3,27)=2.346$, $p=0.095$). These observations are consistent with data that we have published showing the intraperitoneal injection of ethanol increased hypothalamic AgRP IR in ethanol preferring C57BL/6J mice but failed to alter AgRP in moderate ethanol drinking 129/SvJ mice (Cubero et al., 2010).

No changes in α-MSH IR or AgRP IR were detected in other brain regions examined. Importantly, although in some regions the decrease in α-MSH IR did not became apparent until mice had experienced multiple cycles of binge-like ethanol consumption (DMH, ARC, PVN), decreases in α-MSH IR in the LH were detected after a single binge-like drinking cycle, suggesting that melanocortin signaling in this region is critical in the modulation of neurobiological responses associated with binge-like ethanol drinking. These data will be part of a paper that we are currently preparing for publication in which we also show that site-directed infusion of the non-selective melanocortin agonist, melanotan-II (MTII), protects against binge-like ethanol drinking in mice.

For the second goal of Task 1, we performed an additional experiment in which C57BL/6J mice first experienced 0 to 10 4-day binge-like drinking episodes (3 days of rest between episodes). Beginning one week after the last binge-like drinking session, mice began a 40-day 2-bottle (water versus ethanol) voluntary consumption test with concentration ranging from 10 to 20% (v/v) ethanol. Results showed that a prior history of binge-like ethanol drinking significantly increased subsequent voluntary ethanol consumption and preference, effects most robust in groups that initially experienced 6 or 10 binge-like drinking episodes and completely absent in mice that experienced 1 binge-like drinking episode. The subsequent increases of voluntary ethanol consumption and preference that become more robust following repeated episodes of binge-like ethanol drinking may reflect the early stages of ethanol dependence, suggesting that DID procedures may be ideal for studying the transition to ethanol dependence. These data are included in a paper that is currently in press (see the appendix below) in the journal *Alcoholism: Clinical and Experimental Research.*

Task 2: Will determine if binge-like alcohol drinking will be associated with alterations of NeuN, β-endorphin, or POMC protein expression. As a control to determine if the decreases detected in α-MSH IR were potentially secondary to neuronal death stemming from binge-like ethanol drinking, we analyzed the neuronal density in regions that showed decreases in α-MSH IR using NeuN, a marker for neuron-specific nuclear proteins. Representative photomicrographs of NeuN IR in the LH are shown in **Figures 4(B,D)**. Importantly, none of the regions that showed decreases in IR following 1, 3, or 6 cycles of binge-like ethanol drinking (LH, DMH, ARC, and PVN) showed any decreases in neuronal density as measured by NeuN IR (**Figure 6**). Similarly, no significant changes in NeuN IR were detected in sucrose drinking groups in any of the regions examined. Importantly, these results confirm that the reductions detected in α-MSH IR were not likely related to ethanol-induced neuronal loss.

The only regions that we noted significant alterations of β-endorphin IR stemming from a history of binge-like ethanol drinking were in the dorsomedial hypothalamus (DMH) and the dorsomedial regions of the bed nucleus of the stria terminalis (DM BNST; see **Figure 7**). An ANOVA performed on the DMH data achieved statistical significance ($F(3,28) = 3.04$, $p = 0.046$), and planned comparisons showed that relative to the water drinking control group only the group that experienced 6-cycles of binge-like ethanol drinking showed reduced β-endorphin IR in the DMH (**Figure 7, left panel**). An ANOVA performed on the DM BNST data achieved statistical significance ($F(3,16) = 5.24$, $p = 0.01$), and planned comparisons showed that relative to the water drinking control group all binge-like ethanol drinking groups showed increased β-endorphin IR in the DM BNST (**Figure 7, right panel**). Finally, relative to a control group (110.35 ± 3.7), animals that experienced 4 (91.7 ± 5.35) or 18 (75.65 ± 7.11) days of binge-like ethanol exposure exhibited significantly fewer POMC-positive IR cells in the arcuate nucleus of the hypothalamus ($F(2, 27) = 9.74$, $p = 0.001$). Portions of these data will be included in the paper noted above that we are currently preparing and which will also include the α-MSH data described in Task 1.

Task 3: Will determine if the MC3R modulates the effects of MCR agonist on binge-like ethanol drinking in C57BL/6J mice. Administration of MTII, a nonselective MCR agonist, reduces voluntary ethanol consumption in C57BL/6J mice. Previous studies from our lab have demonstrated that central infusion of MTII effectively reduced voluntary ethanol drinking in mutant mice lacking normal expression of MC3R (MC3R-/- mice) but failed to alter ethanol drinking in mice lacking expression of MC4R, demonstrating that central MTII administration reduces voluntary ethanol drinking by signaling through the MC4R. However, evidence shows that the neurocircuitry recruited during excessive binge-like ethanol drinking versus moderate ethanol drinking are not identical. Thus we investigated the potential role of the MC3R in binge-like ethanol intake. To this end, DID procedures, a commonly used animal model of binge-like ethanol drinking and described above, was employed. Wild-type MC3R+/+ and MC3R-/- mice were given intracerebroventricular (i.c.v.) infusion of MTII (0.0, 0.25, 0.50, or 1.0 µg) before 4-hours of access to 20% (v/v) ethanol. Immediately after ethanol access, tail-blood samples were collected to assess blood ethanol concentrations (BECs). Consistent with previous findings, central administration of MTII blunted binge-like ethanol drinking in both MC3R+/+ and MC3R-/- mice (for figures related to this study please see the pre-print by **Olney et al.** in the appendix below). Interestingly, all doses of MTII blunted binge-like ethanol drinking in MC3R-/- mice during the first hour of testing, while only the 1.0 µg dose reduced binge-like drinking in MC3R+/+ mice. Thus, MC3R-/- mice were more sensitive to the protective effects of MTII. These data suggest that MC3Rs oppose the protective effects of MTII against binge-like ethanol drinking, and thus selective MC3R antagonists may have potential therapeutic roles in treating excessive ethanol drinking. In fact, it is possible that MC3R antagonists and MTII agonists could be given in combination to enhance the effectiveness in protecting against excessive alcohol drinking. The Olney et al. paper in the appendix below is currently under review in the journal *Alcohol*.

Task 4: Will determine if the MC3R modulates the effects of MCR antagonist on binge-like ethanol drinking in C57BL/6J mice. MC3R-/- and MC3R+/+ mice were cannulated for testing with DID procedures as described above. On the test day, mice were given i.c.v. infusion of vehicle or a 0.05 or 0.1 µg dose of the MCR antagonist, agouti-related protein (AgRP; n = 12-14/group). Data from this experiment are presented in **Figure 8**. Given the variability between group variance, nonparametric tests of significance were used for statistical analyses (Siegel, 1956). Relative to the vehicle control infusion, the 0.05 dose of AgRP significantly increased ethanol drinking in MC3R+/+ mice ($\chi^2_{(1)} = 4.57$, $p = 0.033$), though the 1.0 µg dose failed to increase binge-like ethanol drinking. These observations are consistent with our previous findings in which a 0.05 µg dose of AgRP was more effective in increasing ethanol intake than higher doses of AgRP (Navarro et al., 2005). On the other hand, neither dose of AgRP increased binge-like ethanol drinking in MC3R-/- mice. These observation suggest that the ability of AgRP to increase binge-like ethanol drinking requires normal MC3R signaling.

Task 5: Will determine if the MC4R modulates the effects of MCR agonist on binge-like ethanol drinking in C57BL/6J mice. MC4R-/- and MC4R+/+ mice were cannulated for testing with DID procedures as described above. On the test day, mice were given infusion of vehicle or a 0.25 or 0.5 µg dose of MTII just before ethanol access (n = 7-13/group). Data from this experiment are presented in **Figure 9**. Relative to the vehicle infusion, the 0.25 µg dose of MTII significantly blunted binge-like ethanol drinking, but only in MC4R+/+ mice ($t(6) = 2.71$, $p = 0.035$). These observations suggest that MTII-induced blunting of binge-like ethanol drinking requires normal MC4R expression, since this drug was ineffective in MC4R-/- mice. We recently reported that MTII-induced reduction of ethanol drinking requires normal MC4R signaling (Navarro et al., 2011). The data from Tasks 4 and 5 will be included together in a future publication.

Task 6: Will determine if the MC4R modulates the effects of MCR antagonist on binge-like ethanol drinking in C57BL/6J mice. MC4R-/- and MC4R+/+ mice were cannulated for testing with DID procedures as described above. On the test day, mice were given infusion of vehicle or a 0.05 or 1.0 µg dose of AgRP just before ethanol access (n = 8/group). Data from this experiment are presented in **Figure 10**. Surprisingly no significant differences emerged in this study, though there was a trend for the 0.05 µg dose to increase ethanol drinking in MC4R+/+ mice. The lack of an effect was surprising since the 0.05 µg dose AgRP increased ethanol drinking in the MC3R+/+ mice described in Task 4, and we have noted that this dose of AgRP increased voluntary ethanol drinking in C57BL/6J mice previously (Navarro et al., 2005). We speculate that a ceiling effect in ethanol drinking may have masked the effects of AgRP in the current study. DID procedures are designed to induce high levels of ethanol drinking in mice, and a comparison of **Figures 8 and 10** shows that at baseline (the vehicle condition) MC4R-/- and MC4R+/+ mice drank more ethanol than MC3R-/- and MC3R+/+ mice. It may be that AgRP could not increase this already high level of ethanol intake.

Task 7: Will determine if MCR agonist and opioid receptor antagonists interact to protect against binge-like alcohol drinking in C57BL/6J mice in an additive, supraadditive (synergistic), or infraadditive interaction. The central polypeptide precursor POMC gives rise to β-endorphin, an endogenous opioid peptide, and the melanocortin (MC) peptides including α-MSH. Opioid receptor antagonists, such as naltrexone (NAL), have been demonstrated to reduce ethanol consumption in rodents, and a growing body of evidence indicates that MC

receptor agonists blunt ethanol intake (Navarro et al., 2005; Navarro et al., 2003). Interestingly, central opioid and MC pathways have been demonstrated to interact in their modulation of nociception and feeding behavior (Ercil et al., 2005; Greenway et al., 2009; Starowicz et al., 2002). Since opioids and MC peptides modulate ethanol consumption, the goal of the present work was to determine if compounds aimed at opioid or MC receptors, when presented in combination, interact additively or synergistically in the modulation of binge-like ethanol drinking in C57BL/6J mice. We used DID procedures and first established the effects of intraperitoneally (i.p.) injected MC agonist MTII (**Figure 11**) or NAL (**Figure 12**) on binge-like ethanol drinking and associated BECs using a range of doses (0, 0.3, 3.0, and 10 mg/kg for each drug). ANOVAs performed on MTII data revealed the MTII dose-dependently blunted binge-like ethanol drinking [$F(4, 42) = 13.78$, $p < 0.001$] and associated BECs [$F(4, 42) = 6.57$, $p < 0.001$], and post hoc Tukey's tests showed that relative to the saline injected control group, the 1 3, and 10 mg/kg doses of MTII significantly blunted binge-like ethanol drinking and associated BECs. Similarly, ANOVAs performed on NAL data revealed that NAL dose-dependently blunted binge-like ethanol drinking [$F(4, 40) = 7.42$, $p < 0.001$] and associated BECs [$F(4, 40) = 2.81$, $p = 0.038$], and post hoc tukey's tests showed that the 3 and 10 mg/kg doses of NAL blunted binge-like ethanol drinking and associated BECs relative to the saline injected control group.

Next, based on the dose-response data we established the ED_{20} and ED_{30} for each drug, and then combined the low (ED_{20}) and high (ED_{30}) dose of each drug with the dose-response range of the other drug. Results showed that MTII was 3.4-fold more potent than NAL in blunting binge-like ethanol drinking (based on ED_{50} values). MTII was also more effective, as the 10 mg/kg dose of MTII produced a 72% reduction of binge-like ethanol drinking while this same dose of NAL reduced drinking by only 49%. When administered in combination, the low ED_{20} (but not the ED_{30}) dose of MTII (0.26 mg/kg) shifted the NAL dose-response curve to the left by a factor of 7 (i.e., NAL was 7-fold more potent when administered in combination with MTII relative to when it was administered alone; see **Figure 13**). Subsequent isobolographic analyses (n = 10 to 12 group) of these data showed that MTII synergistically augmented the ability of NAL to blunt binge-like ethanol drinking (**Figure 14**). In this figure, the perpendicular line intersecting the naltrexone ED_{50} and the MTII ED_{50} represents the theoretical line of additivity. Vertical lines represent the 95% confidence level (C.L.) of the ED_{50} values for either NAL or MTII: when the ED_{50} was to the left of the theoretical line of additivity and the C.L. lines did not overlap the area encompassed by the dotted lines, the interaction was considered to be supra-additive, whereas when the C.L. lines did overlap with that area, the interaction was considered additive. As seen in **Figure 14**, the 0.26 mg/kg dose of MTII significantly shifted the NAL ED_{50} to the left (beyond the C.L. region), indicating that MTII synergistically potentiated the ability of NAL to blunt binge-like ethanol drinking. These observations suggest that MC receptor agonists may improve the therapeutic effectiveness of NAL in the treatment of alcohol abuse disorders when these drugs are given in combination. A manuscript based on data from this Task is currently in preparation and will be submitted in the near future.

Task 8: Will determine if combining maximally effective doses of MTII and naltrexone will produce effects greater than those observed for either drug alone. For this experiment, mice were tested with DID procedures as described above, and first given i.p. injection of MTII or NAL alone. The left panel of **Figure 15** shows selected doses of MTII and NAL presented alone, and the right panel of **Figure 15** shows data when MTII and NAL were presented in combination. The analysis revealed that the combination of 3.0 mg/kg NAL and 3.0 mg/kg MTII produced a 75% decrease in ethanol consumption, which was only slightly larger than the 62% decrease associated the 3.0 mg/kg dose of MTII alone. Similar effects were obtained with the other combinations tested, suggesting that the maximal effects produced by the combination of

high doses of naltrexone and MTII were not greater than those produced by the highest dose (10 mg/kg) of these drugs when administered alone

KEY RESEARCH ACCOMPLISHMENTS

- Established that repeated bouts of binge-like ethanol drinking are associated with significant attenuation of α-MSH immunoreactivity in brain regions implicated in the modulation of neurobiological responses to ethanol.

- Established that repeated bouts of binge-like ethanol drinking promote subsequent increases of voluntary ethanol drinking, consistent with a dependence-like phenotype.

- Established that repeated bouts of binge-like ethanol drinking are associated with significant increases of agouti-related protein (AgRP; a natural MCR antagonist) immunoreactivity in brain regions implicated in the modulation of neurobiological responses to ethanol. As AgRP is a natural melanocortin receptor antagonist, these findings are directly relevant to the present project.

- Established that a history of binge-like ethanol consumption is associated with a significant reduction of β-endorphin immunoreactivity in regions of the hypothalamus, but a significant increase of β-endorphin immunoreactivity in the bed nucleus of the stria terminalis.

- Established that binge-like ethanol exposure is associated with a significant reduction of POMC immunoreactivity in the arcuate nucleus of the hypothalamus.

- Established that a history of binge-like ethanol drinking does not alter NeuN immunoreactivity in the brains of mice, confirming that changes in protein levels noted above are not likely secondary to ethanol-induced neuronal death.

- Established that the protective effects of the MCR agonist MTII in reducing binge-like ethanol drinking are enhanced in mice lacking the MC3R. These observations suggest that blockade of the MC3R with selective receptor antagonists may have therapeutic value for protecting against excessive binge alcohol drinking.

- Established that MCR antagonist-induced increases of ethanol drinking require normal expression of the MC3R in the brain.

- Establishing the melanocortin receptor agonist MTII protects against binge-like ethanol drinking via the MC4R. These observations suggest that MC4R agonists may have therapeutic value for treating excessive binge alcohol drinking.

- Established that the opioid receptor antagonist naltrexone and the MCR agonist MTII dose-dependently protect against binge-like ethanol drinking in mice.

- Established that a low dose of the MCR agonist MTII synergistically increased the ability of naltrexone to blunt binge-like ethanol drinking in mice. Thus, the inclusion of MCR

agonists may improve the effectiveness of naltrexone-based therapies in the treatment of alcohol abuse disorders.

- Established that combining the maximally effective doses of MTII and naltrexone did not produced effects on binge-like ethanol drinking greater than those observed for either drug alone.

REPORTABLE OUTCOMES:

PERSONNEL SUPPORTED BY THIS GRANT: Note that in all references listed in this section below, Todd E. Thiele (Thiele, T. E.), Montserrat Navarro (Navarro, M.), and Mitchell J. Picker (Picker, M. J.) received pay from this grant for research effort. All other personnel listed received salaries from other sources.

PUBLICATONS STEMMING FROM GRANT TASKS:

1. Cox, B. R., Olney, J. J., Lowery-Gionta, E. G., Sprow, G. M., Rinker, J. A., Navarro, M., Kash, T. L., & Thiele, T. E. (in press). Repeated cycles of binge-like ethanol drinking in male C57BL/6J mice augments subsequent voluntary ethanol intake but not other dependence-like phenotypes. *Alcoholism: Clinical & Experimental Research.*

2. Olney, J. J., Sprow, G. M., Navarro, M., & Thiele, T. E. (under review). The protective effects of the melanocortin agonist, melanotan-II (MTII), against binge-like ethanol drinking are facilitated by deletion of the MC3 receptor in mice. *Alcohol.*

3. Navarro, M., Lerma-Cabrera, J. M., Carvajal, F., Lowery, E. G., Cubero, I., & Thiele, T. E. (2011). Assessment of voluntary ethanol consumption and the effects of a melanocortin (MC) receptor agonist on ethanol intake in mutant C57BL/6J mice lacking the MC-4 receptor. *Alcoholism: Clinical & Experimental Research, 35,* 1058-1066.

4. Cubero, I., Navarro, M., Carvajal, F., Lerma-Cabrera, J. M., & Thiele, T. E. (2010). Ethanol-induced increase of agouti-related protein (AgRP) immunoreactivity in the arcuate nucleus of the hypothalamus of C57BL/6J, but not 129/SvJ, inbred mice. *Alcoholism: Clinical & Experimental Research, 34* 693-701.

PUBLISHED ABSTRACTS STEMMING FROM GRANT TASKS:

1. Navarro, N., Picker, M. J., & Thiele, T. E. (2012). A low dose of the melanocortin agonist MTII synergistically augments naltrexone-induced attenuation of binge-like ethanol drinking in C57BL/6J mice. *Alcoholism: Clinical & Experimental Research, 36,* 265A.

2. Olney, J., Sprow, G. M., & Thiele, T. E. (2012). The melanocortin (MC) receptor agonist, melanotan-II, blunts binge-like ethanol drinking in mice via the MC-4 receptor (MC4R). *Society for Neuroscience Abstracts, Online.*

3. Sprow, G. M., & Thiele, T. E. (2012). Melanocortin receptor signaling in the lateral hypothalamus modulates binge-like ethanol consumption in C57BL/6J mice. *Society for Neuroscience Abstracts, Online.*

4. Sprow, G. M., Lowery, E. G., Lyons, A. M., Navarro, M., & Thiele, T. E. (2011). Repeated binge-like ethanol intake in C57BL/6J mice leads to decreased α-MSH immunoreactivity (IR) and increased AgRP in key brain regions. *Alcoholism: Clinical & Experimental Research, 35,* 246A

5. Navarro, M., Lerma-Cabrera, J. M., Carvajal, F., Lowery, E. G., Cubero, I., & Thiele, T. E. (2010). Central, but not peripheral, administration of melanocortin (MC) receptor agonists require the MC-4 receptor to reduce ethanol intake. *Alcoholism: Clinical & Experimental Research, 34,* 15A.

INVITED PRESENTATIONS COVERING WORK RELATED TO THIS GRANT:

1. Alcohol and Drug Abuse Research Program and Translational Addiction Research Center, Washington State University, Pullman, Washington (March, 2013). Talk titled *Overlapping Neuropeptide Modulation of Binge Alcohol Drinking and Eating Disorders: Can We Kill Two Birds with One Stone?*

2. Bowles Center for Alcohol Studies, University of North Carolina, Chapel Hill, North Carolina (September, 2012). Talk titled *Melanocortin Signaling in the Lateral Hypothalamus Modulates Binge-Like Ethanol Drinking.*

3. Departamento de Psicologia Experimental y Fisiologia del Comportamiento, University of Granada, Granada, Spain (June, 2010). Talk titled *A Role for Central Neuropeptides in Binge Alcohol Drinking*.

4. Departamento de Neurociencia y Ciencias de la Salud, University of Almeria, Almeria, Spain (April, 2010). Talk titled *A Role for Central Neuropeptides in Binge Alcohol Drinking*.

CONCLUSIONS: We have made numerous important discoveries from the tasks and experiments that were supported by this grant. We have discovered that binge-like ethanol drinking in mice promotes reductions of α-MSH IR, and significant increases of AgRP IR. Since α-MSH is protective against binge drinking, and AgRP promotes binge drinking, these observations show that the MC system becomes compromised over the course of binge drinking, and we speculate that this promotes continued excessive ethanol drinking. Since we also found that a history of binge-like drinking promotes subsequent increases of overall voluntary ethanol drinking, consistent with a dependence-like state, it may be that binge-induced changes in the MC system reflect plasticity in the brain the contributes to alcohol dependence. Importantly, we found that MCR agonists can protect against binge drinking by acting on the MC4R. Thus, MC4R agonists may have therapeutic value. On the other hand, since the MCR agonist was more effective in mice lacking the MC3R, we speculate that MC3R antagonists may hold therapeutic value for treating alcohol abuse disorders. One very exciting possibility is that a

combined therapy involving MC4R agonists and MC3R antagonists my increase positive outcomes in treating alcohol abuse disorders. We have established that the opioid receptor antagonist naltrexone and MCR agonist MTII protect against binge-like ethanol drinking in a dose-dependent manner. Importantly, consistent with our hypothesis, we have found that the MCR agonist MTII synergistically augments the ability of the opioid receptor antagonist naltrexone to protect against binge-like drinking in mice. Since naltrexone is currently an FDA approved treatment for alcohol abuse disorders, our results suggest that combining a MC4R agonist with naltrexone may be a way to significantly improve an existing pharmacotherapy. So what does this mean? These results have important implications for possible pharmacological medical treatment of binge drinking in the human population. Specifically, melanocortin receptor agonists aimed at the MC4R (or antagonists aimed at the MC3R), as well a opioid receptor antagonist, may prevent binge drinking in at-risk individuals, and thus protect these people from the negative behavioral and biological consequences of regular binge drinking. Importantly, preventing frequent binge drinking will reduce the risk of future alcohol abuse disorders and dependence. These findings may be considered of high relevance to the U.S. military given the high prevalence of binge drinking in the military population.

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APPENDICES:

- Figures 1-15. In figures, * indicates significant differences from the control group condition at the $p < 0.05$ level.

- 4 papers that were supported by this grant. Navarro et al. (2011) and Cubero et al. (2010) are in print, Cox et al. (2013) is in press, and the Olney et al. paper is currently under review.

- Copies of published conference abstracts describing research supported by this grant.

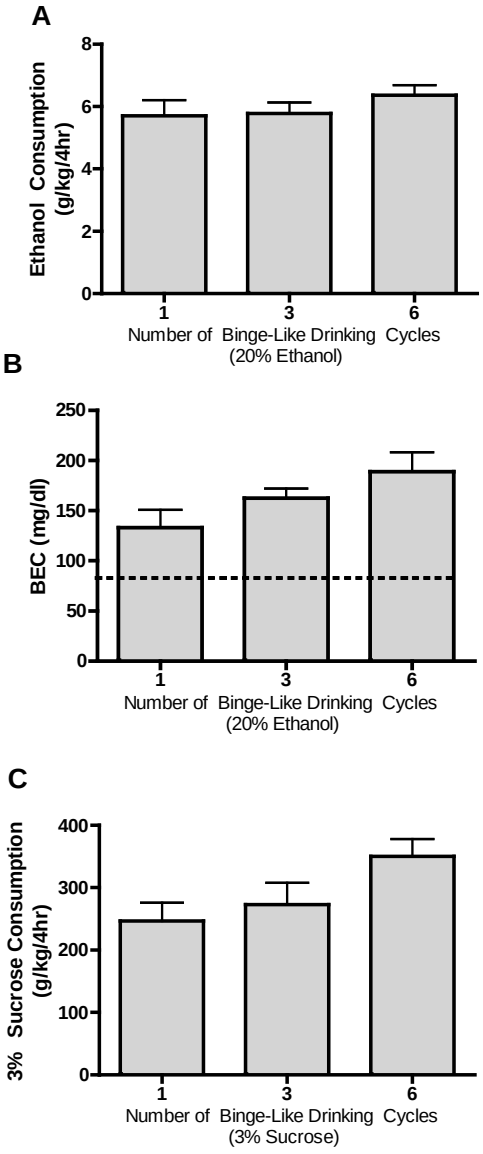

Figure 1: Total fluid consumed on the final day of binge-like 20% ethanol (A) or 3% sucrose (C) drinking did not differ between mice that experienced 1, 3, or 6 cycles binge-like drinking. BECs from the final session of binge-like ethanol drinking (B) did not differ between mice that experienced 1, 3, or 6 cycles of binge-like ethanol drinking. All data are shown as mean ± SEM, and significance was accepted at $p<0.05$. Dashed line (B) represents 80mg/dl, defined by NIAAA as the BEC required for a 'binge.'

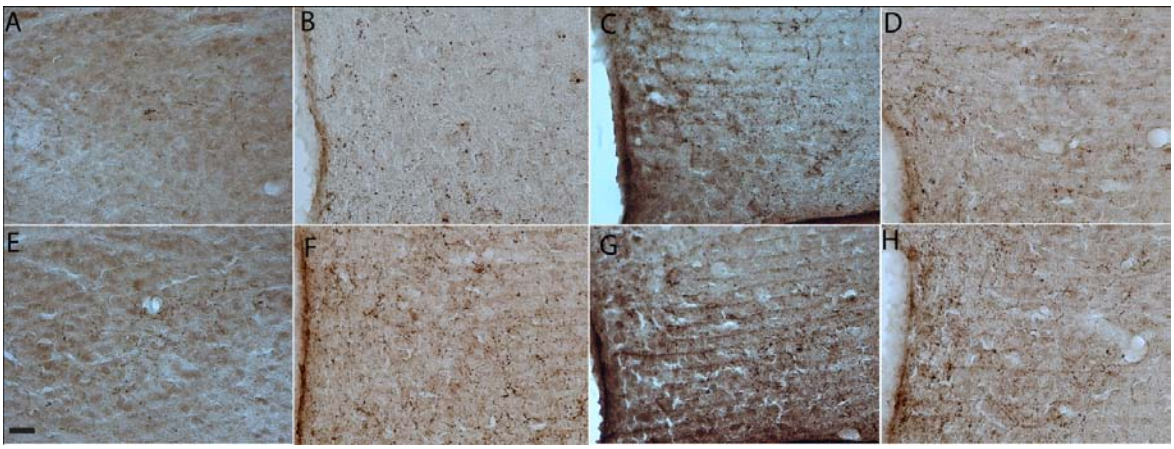

Figure 2: Representative photomicrographs showing α-MSH IR (% total area) in the lateral hypothalamus (A, E), dorsomedial hypothalamus (B, F), arcuate nucleus (C, G) and paraventricular nucleus (D, H). The top row are from mice that experienced 6 cycles of binge-like ethanol consumption, and the bottom row are from mice that consumed only water for the duration of the experiment. Images were captured at a magnification of 40x, and the scale bar = 50 μm.

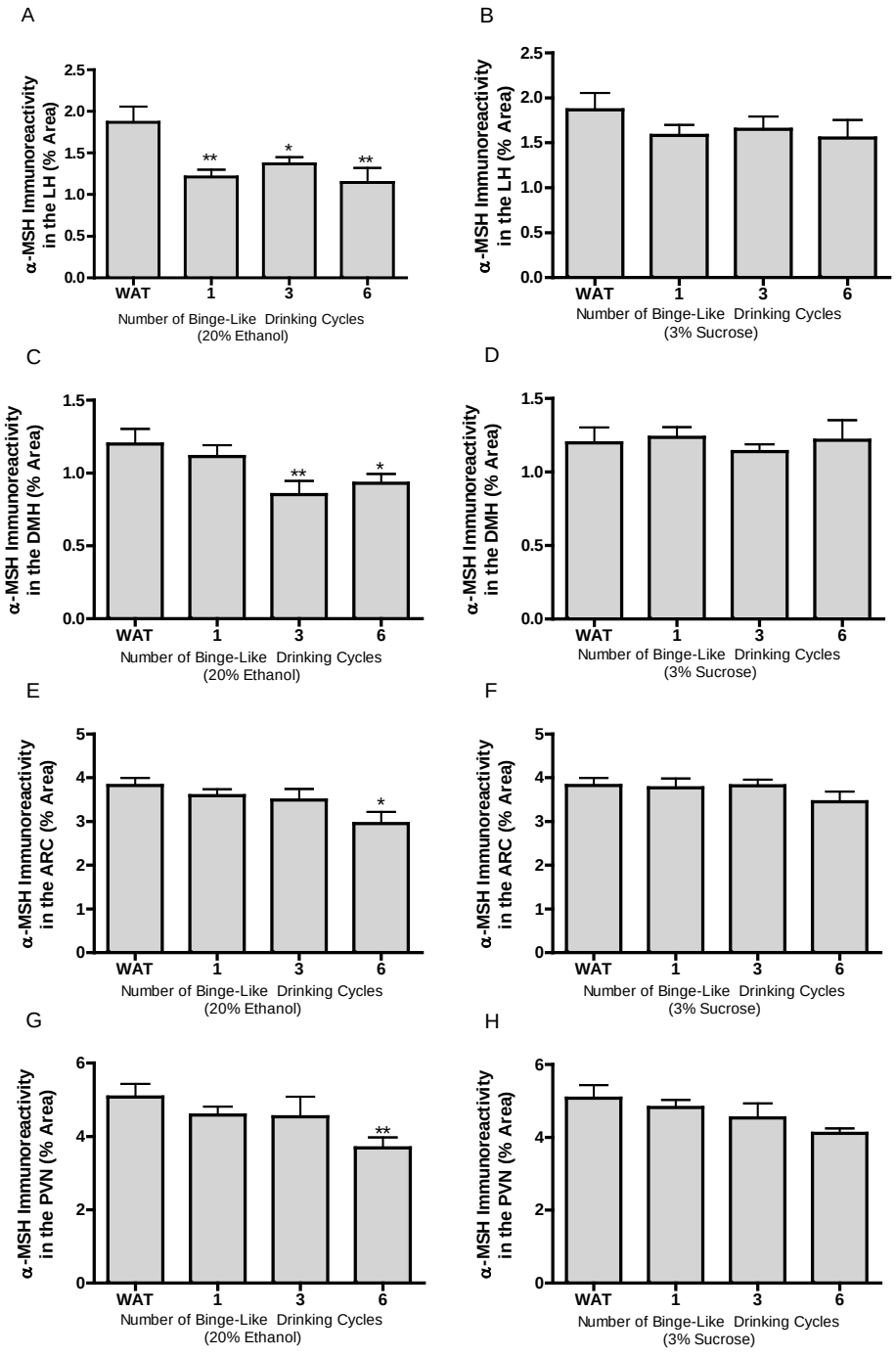

Figure 3: Binge-like consumption of 20% ethanol, but not 3% sucrose, significantly reduced α-MSH IR in the LH (A), DMH (C), ARC (E) and PVN (G). The left column (A,C,E, G) represents α-MSH IR from mice that experienced 1, 3, or 6 cycles of binge-like 20% ethanol consumption, and the right column (B, D, F, H) represents mice that experienced 1, 3, or 6 cycles of binge-like 3% sucrose consumption. All data are shown as mean ± SEM, and significance was accepted at $p<0.05$. *denotes significance of $p<0.05$ vs. WAT, ** denotes $p<0.01$ vs. WAT.

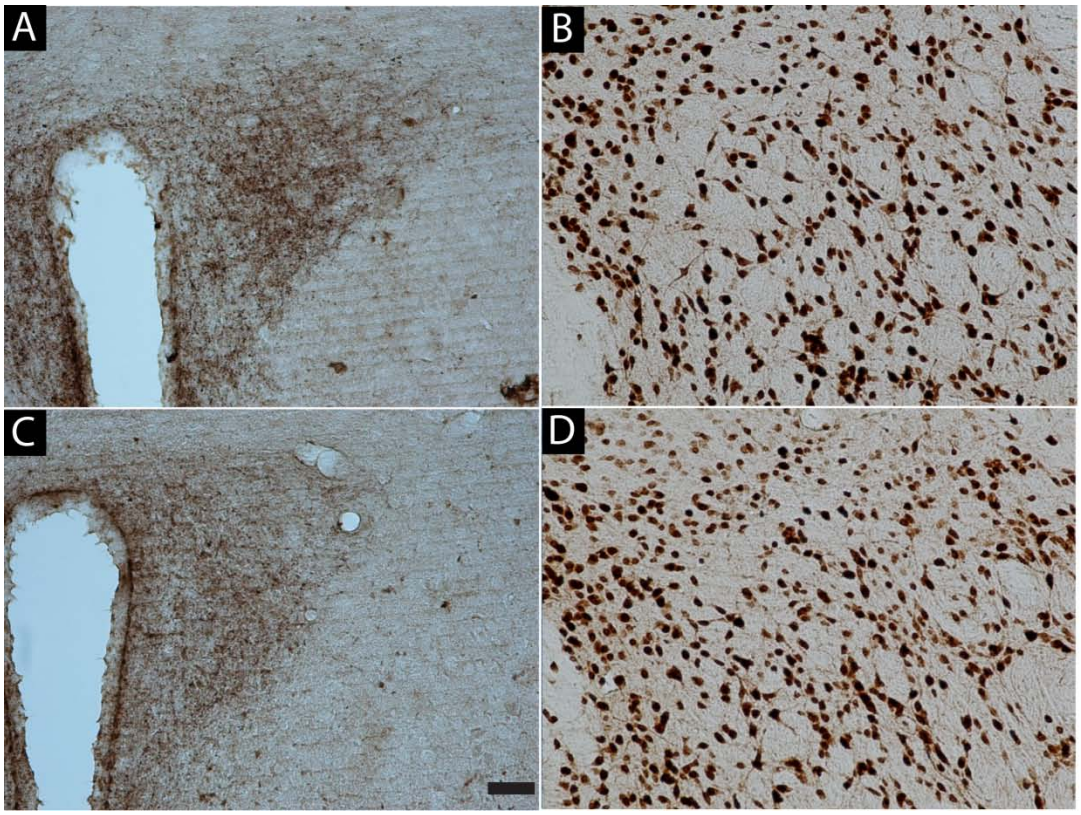

Figure 4: Representative photomicrographs showing AgRP IR in the paraventricular nucleus (PVN) and NeuN IR in the lateral hypothalamus (LH). The top row depicts mice that experienced 6 cycles of binge-like ethanol consumption, and the bottom row depicts mice that consumed only water for the duration of the experiment. Images were captured at a magnification of 20x, and the scale bar = 100 μm.

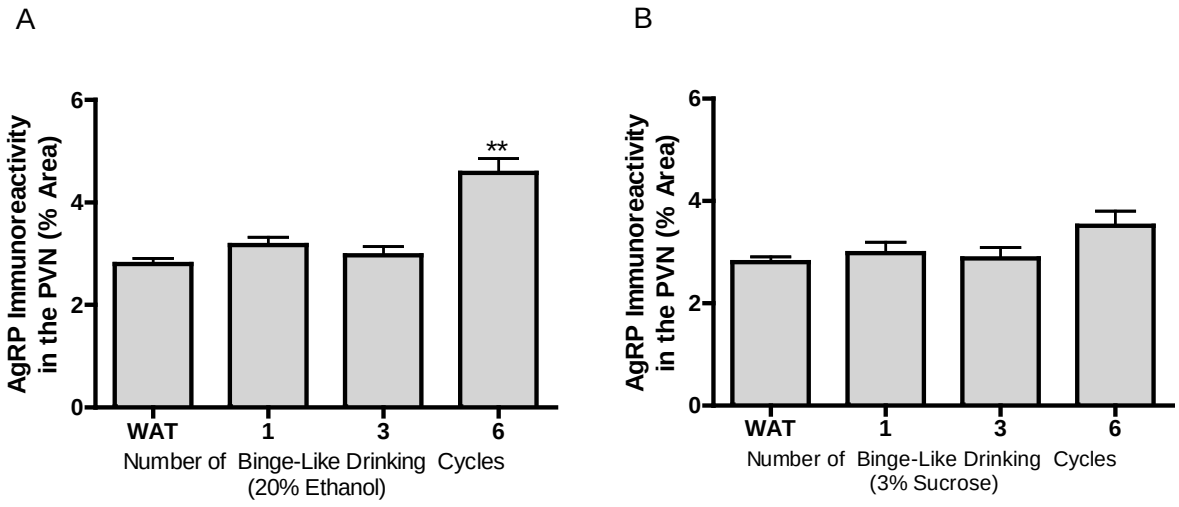

Figure 5: Binge-like consumption of 20% ethanol (A), but not 3% sucrose (B), significantly increased AgRP IR in the PVN. All data are shown as mean ± SEM, and significance was accepted at $p<0.05$. ** denotes $p<0.01$ vs. WAT.

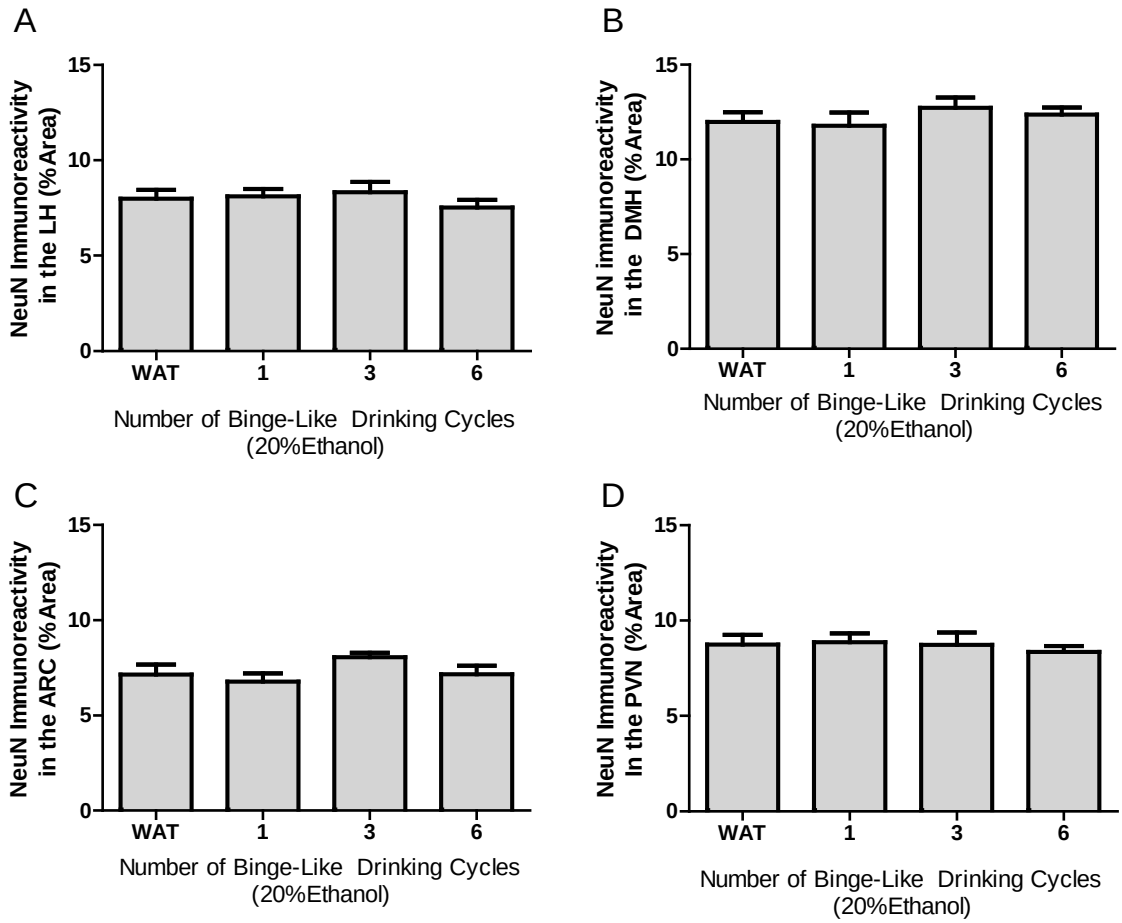

Figure 6: Binge-like consumption of 20% ethanol did not significantly affect NeuN IR in the LH (A), DMH (B), ARC (C), or PVN (D). All data are shown as mean ± SEM, and significance was accepted at $p<0.05$.

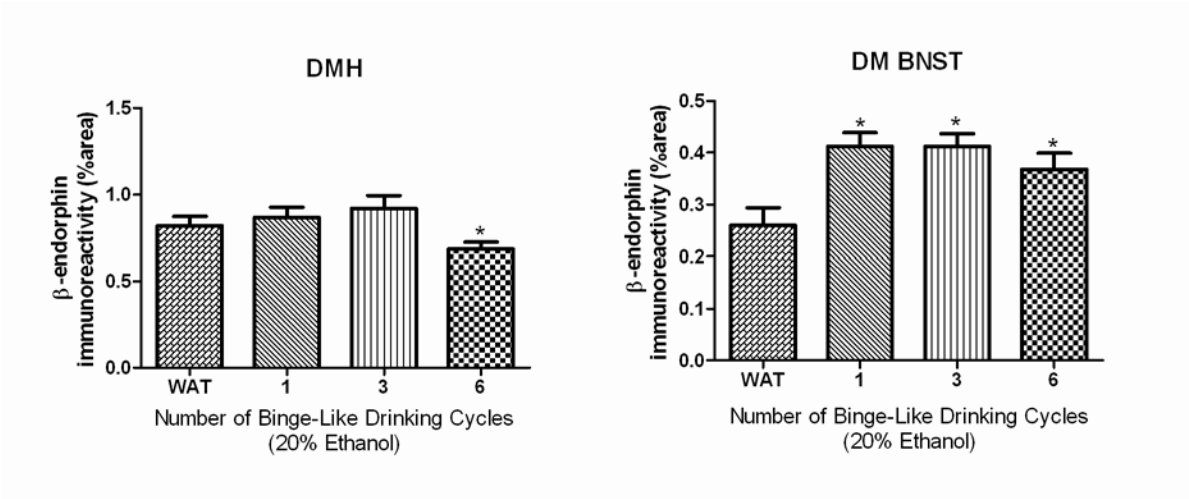

Figure 7: Binge-like consumption of 20% ethanol significantly reduced β-endorphin immunoreactivity in the dorsomedial hypothalamus (DMH) after 6-cycles of binge-like ethanol drinking (left panel) but significantly increased β-endorphin immunoreactivity in the dorsomedial bed nucleus of the stria terminalis (DM BNST) after 1-, 3-, and 6-cycles of binge-like ethanol drinking (right panel). All data are shown as mean ± SEM, and significance was accepted at $p<0.05$. ** denotes $p<0.01$ vs. WAT.

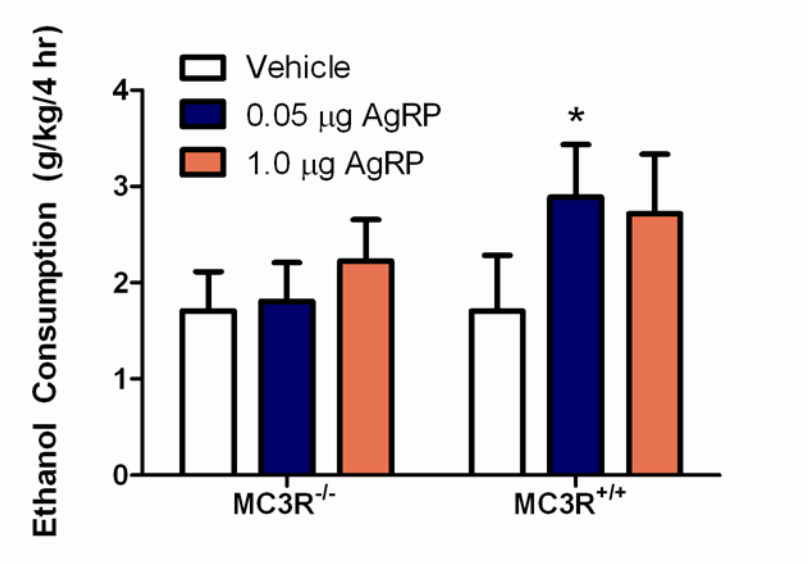

Figure 8: : Binge-like ethanol consumption in mutant mice lacking the MC3R (MC3R-/-) or wild-type mice (MC3R+/+) following i.c.v. infusion of vehicle or the non-selective MCR antagonist AgRP. Administration of AgRP significantly increased binge-like ethanol drinking at the 0.05 μg dose in MC3R+/+, but not MC3R-/-, mice. Data are presented as mean ± SEM. * $p < 0.05$ relative to vehicle.

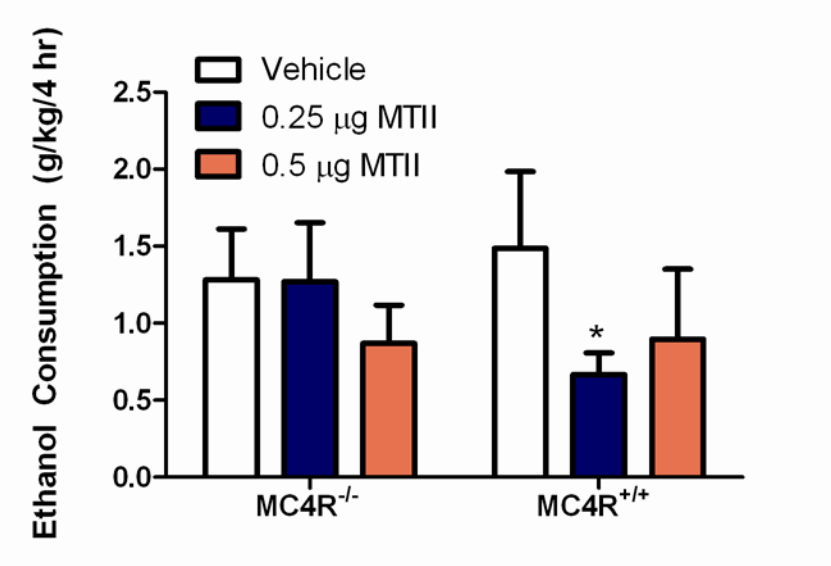

Figure 9: : Binge-like ethanol consumption in mutant mice lacking the MC4R (MC4R-/-) or wild-type mice (MC4R+/+) following i.c.v. infusion of vehicle or the non-selective MCR agonist MTII. MTII significantly attenuated binge-like ethanol drinking in wild-type MC4R+/+ mice at the 0.25 μg dose but failed to alter ethanol drinking in MC4R-/-mice, indicating that MTII-induced blunting of binge-like ethanol drinking requires the MC4R. Data are presented as mean ± SEM. * $p < 0.05$ relative to vehicle.

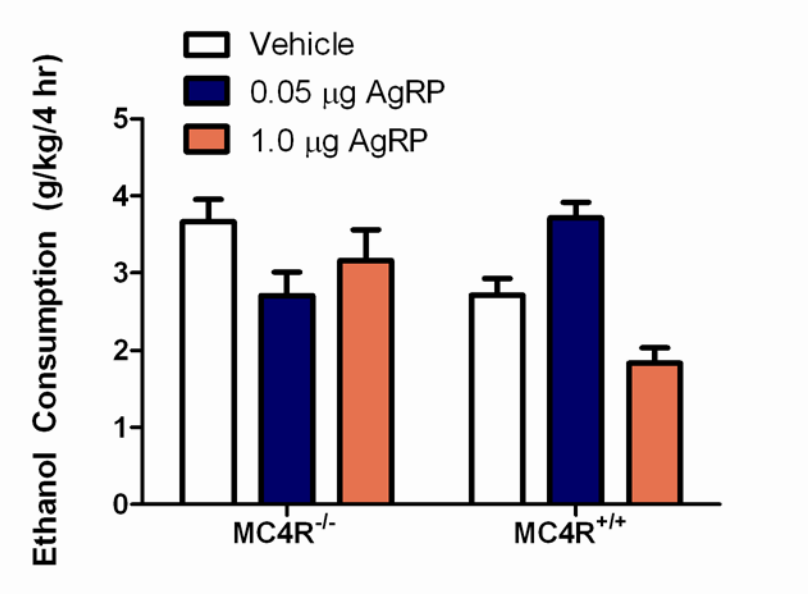

Figure 10: : Binge-like ethanol consumption in mutant mice lacking the MC4R (MC4R-/-) or wild-type mice (MC4R+/+) following i.c.v. infusion of vehicle or the non-selective MCR antagonist AgRP.

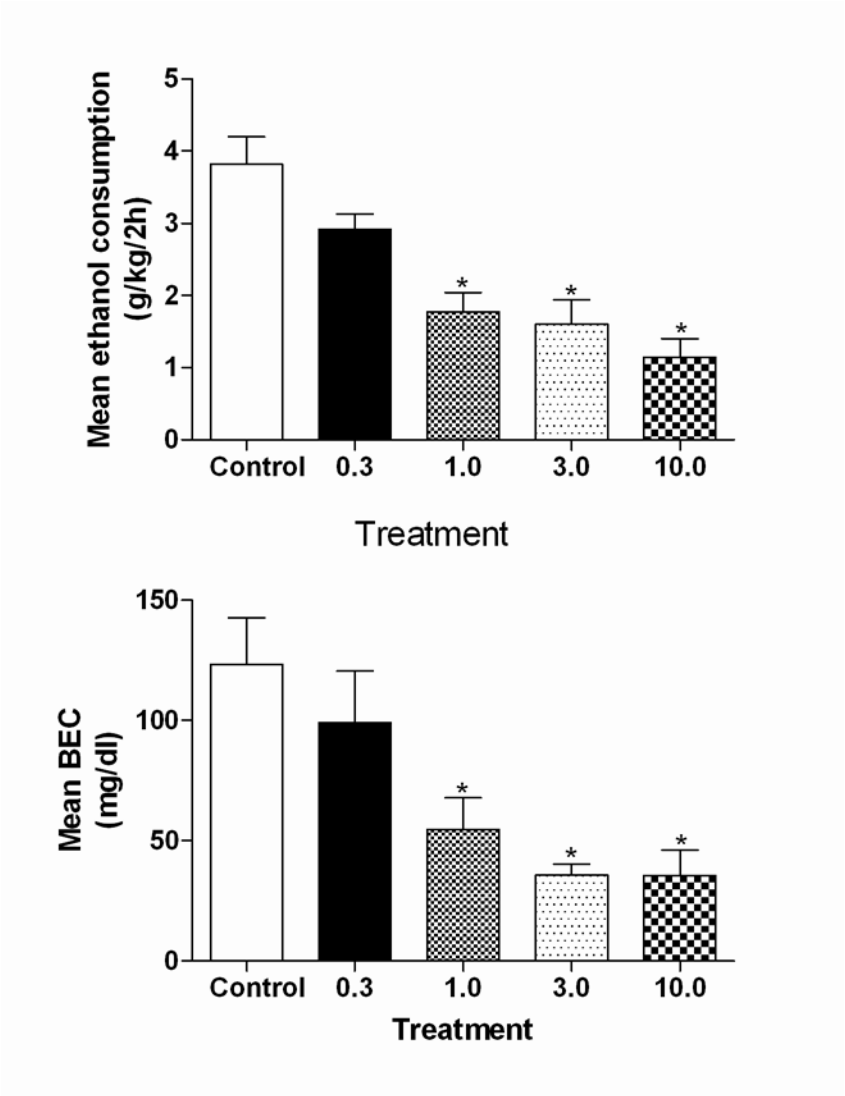

Figure 11: Dose-response assessment of intraperitoneally injected MTII (mg/kg) on 2-hour binge-like ethanol drinking (top panel) and associated blood ethanol levels (BECs; bottom panel) in C57BL/6J mice (n = 9-10/group). ANOVAs revealed that MTII dose-dependently blunted binge-like ethanol drinking and associated BECs. Data are presented as mean $\pm$ SEM. *$p < 0.05$ relative to the saline-injected group (Control).

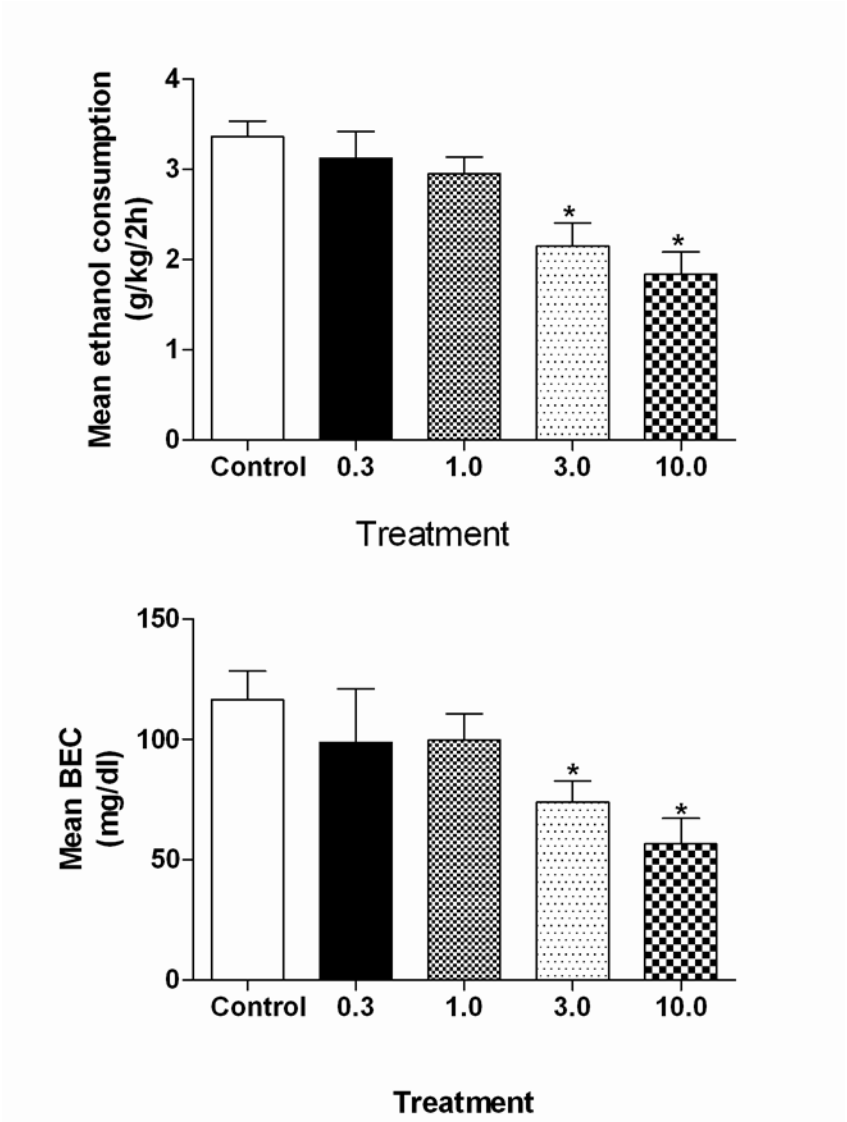

Figure 12: Dose-response assessment of intraperitoneally injected naltrexone (NAL; doses represent mg/kg) on 2-hour binge-like ethanol drinking (top panel) and associated blood ethanol levels (BECs; bottom panel) in C57BL/6J mice (n = 9-10/group). ANOVAs revealed that NAL dose-dependently blunted binge-like ethanol drinking and associated BECs. Data are presented as mean ± SEM. *$p < 0.05$ relative to the saline-injected group (Control).

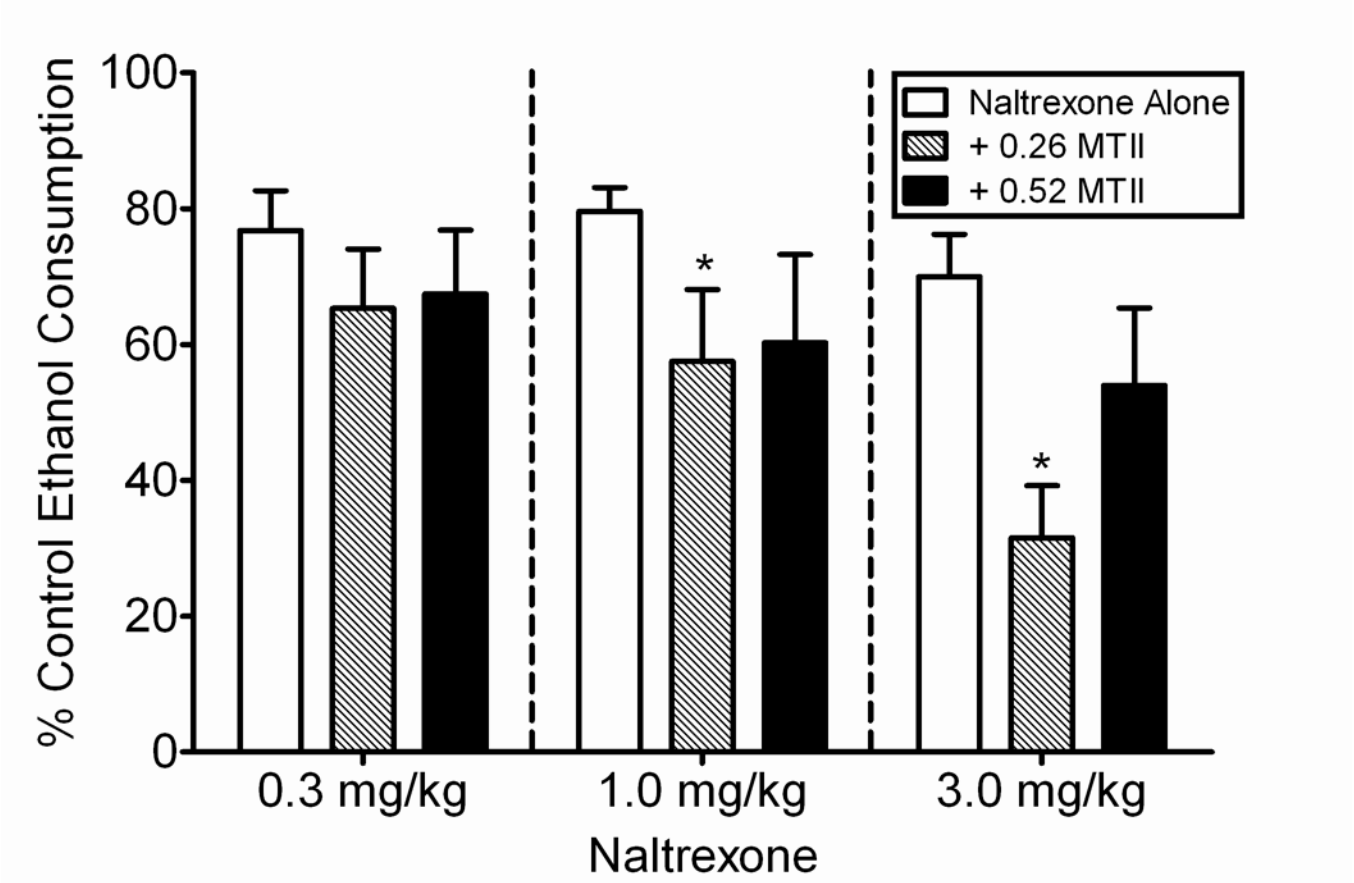

Figure 13: Effects of naltrexone alone and in combination with the approximate ED_{20} (0.26 mg/kg) and ED_{30} (0.52 mg/kg) doses of MTII. These doses of MTII were based on a preliminary assessment of its relative potency. Doses of naltrexone were tested in 9-14 mice and the combination with MTII in 8-11 mice. Ordinate: % ethanol consumption expressed as % mean levels of ethanol consumption obtained during the 3 baseline days prior to testing. Abscissa: dose of naltrexone alone or in combination with MTII expressed in mg/kg. Vertical bars represent the standard error of the mean. * $p < 0.05$ relative to naltrexone alone.

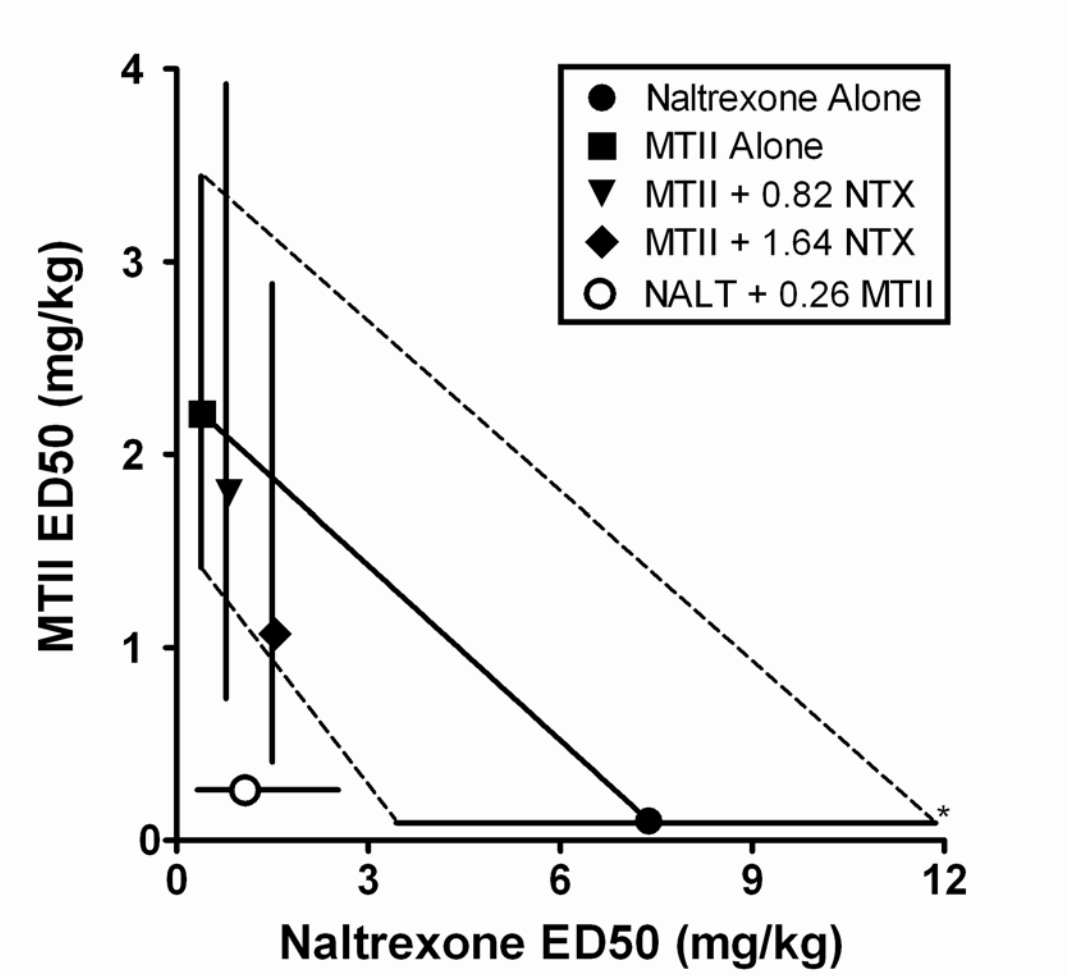

Figure 14: Isobolograms for naltrexone and MTII in combination on ethanol consumption. Abscissa: ED_{50} value (95% C.L.) for naltrexone expressed in mg/kg. Ordinate: ED_{50} value (95% C.L.) for MTII expressed in mg/kg. The perpendicular line intersecting the naltrexone ED_{50} and the MTII ED_{50} represent the theoretical line of additivity. Vertical lines represent the 95% C.L. of the ED_{50} values for either naltrexone or MTII. For drug combinations, when the ED_{50} was to the left of the theoretical line of additivity and the C.L. lines did not overlap the area encompassed by the dotted lines, the interaction was considered to be supra-additive, whereas when the C.L. lines did overlap with that area, the interaction was considered additive. Thus, the 0.26 mg/kg dose of MTII shifted the ED50 of naltrexone to the left to a point considered to be supra-additive (synergistic).

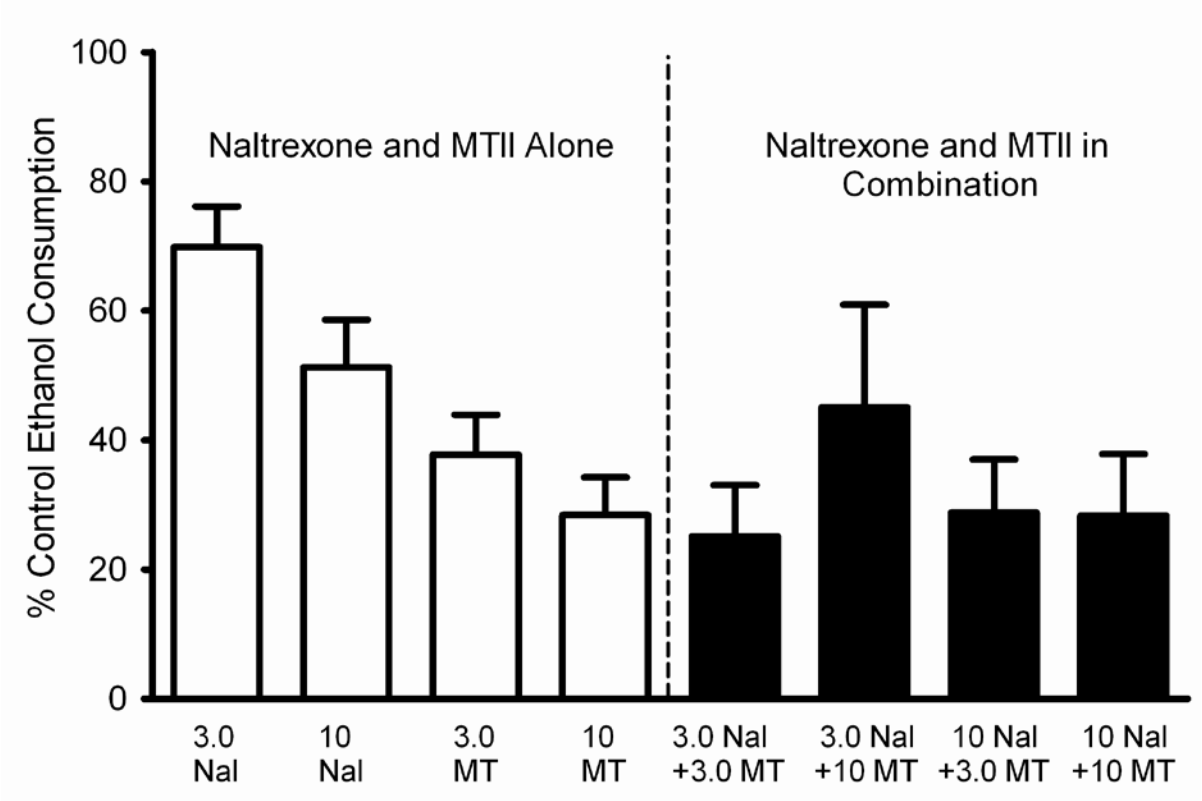

Figure 15: Effects of selected doses of naltrexone and MTII administered alone (left) and in selected combinations (right) on ethanol consumption. The two doses (3.0 and 10 mg/kg) of naltrexone and MTII selected for analysis produced the largest decreases in ethanol consumption when administered alone. When administered alone, doses of naltrexone and MTII were tested in 9-14 mice, whereas the combination data are based on 7-10 mice. Ordinate: % ethanol consumption expressed as % mean levels of ethanol consumption obtained during the 3 baseline days prior to testing. Abscissa: doses of naltrexone and MTII alone and in combination expressed in mg/kg. Vertical bars represent the standard error of the mean.

Repeated Cycles of Binge-Like Ethanol Drinking in Male C57BL/6J Mice Augments Subsequent Voluntary Ethanol Intake But Not Other Dependence-Like Phenotypes

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Running Title: Repeated Binge-Like Drinking

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ABSTRACT

Background: Recently, procedures have been developed to model specific facets of human alcohol abuse disorders, including those that model excessive binge-like drinking (i.e., “drinking in the dark”, or DID procedures) and excessive dependence-like drinking (i.e., intermittent ethanol vapor exposure). Similar neuropeptide systems modulate excessive ethanol drinking stemming from both procedures, raising the possibility that both paradigms are actually modeling the same phenotypes and triggering the same central neuroplasticity. Therefore, the goal of the present project was to study the effects of a history of binge-like ethanol drinking, using DID procedures, on phenotypes that have previously been described with procedures to model dependence-like drinking.

Methods: Male C57BL/6J mice first experienced 0 to 10 4-day binge-like drinking episodes (3 days of rest between episodes). Beginning 24-h after the final binge-like drinking session, mice were tested for anxiety-like behaviors (with elevated plus maze (EPM) and open-field locomotor activity tests), ataxia with the rotarod test, and sensitivity to handling-induced convulsions (HICs). One week later, mice began a 40-day 2-bottle (water versus ethanol) voluntary consumption test with concentration ranging from 10 to 20% (v/v) ethanol.

Results: A prior history of binge-like ethanol drinking significantly increased subsequent voluntary ethanol consumption and preference, effects most robust in groups that initially experienced 6 or 10 binge-like drinking episodes and completely absent in mice that experienced 1 binge-like drinking episode. Conversely, a history of binge-like ethanol drinking did not influence anxiety-like behaviors, ataxia, or HICs.

Conclusions: Excessive ethanol drinking stemming from DID procedures does not initially induce phenotypes consistent with a dependence-like state. However, the subsequent increases of voluntary ethanol consumption and preference that become more robust following repeated

episodes of binge-like ethanol drinking may reflect the early stages of ethanol dependence, suggesting that DID procedures may be ideal for studying the transition to ethanol dependence.

Key Words: ethanol; binge-like; dependence; C57BL/6J; anxiety-like; ataxia; seizure.

INTRODUCTION

Early pre-clinical alcoholism research primarily relied on animal models that involved voluntary consumption of ethanol, in which rats or mice were given 24-h/day access to ethanol and water simultaneously in separate bottles. However, voluntary ethanol consumption may not be the most appropriate model for human alcohol abuse disorders, as rodents typically consume low amounts of ethanol that do not generate blood ethanol levels thought to be pharmacologically meaningful. Recently, procedures have been developed to model specific facets of human alcohol abuse disorders. Such procedures include those that model excessive binge-like drinking prior to the development of dependence (Boehm et al., 2008; Lowery-Gionta et al., 2012; Lowery et al., 2010; Sparrow et al., 2012; Sprow and Thiele, 2012), excessive ethanol intake stemming from ethanol dependence (Funk et al., 2007; Gilpin et al., 2011; Roberts et al., 1996), and excessive relapse-like ethanol drinking (Spanagel and Holter, 2000; Spanagel et al., 1996; Sparta et al., 2009) and ethanol seeking behaviors (Le et al., 1999; Le et al., 1998; Weiss and Liu, 2002). These models have been useful for discovering the neurochemical pathways and the neurocircuitry involved in alcohol-related behaviors.

Recent evidence has been presented suggesting that neuropeptide systems, specifically neuropeptide Y (NPY) and corticotropin releasing factor (CRF), modulate excessive binge-like ethanol drinking in C57BL/6J mice that have been described as non-dependent. Using the procedure called “drinking in the dark” (DID; (Rhodes et al., 2005; Rhodes et al., 2007)), central administration of CRF-1 receptor (CRF1R) antagonists and NPY were found to prevent binge-like ethanol drinking in mice. Interestingly, CRF1R antagonists and NPY failed to alter low level non-binge-like ethanol drinking, suggesting that central CRF and NPY systems are recruited only after sufficient blood/brain ethanol concentrations are achieved, which may motivate continued binge-like drinking (Lowery-Gionta et al., 2012; Lowery et al., 2010; Sparrow et al., 2012). Interestingly, central administration of CRF1R antagonists or NPY have also been shown

to protect against excessive dependence-like ethanol drinking in rodents that have had a prior history of repeated intermittent ethanol vapor exposure, but these compounds failed to alter low level ethanol intake in non-dependent animals (Gilpin et al., 2011; Roberto et al., 2010). The striking similarity between results obtained with models of binge-like ethanol drinking in "non-dependent" animals and in "dependent" animals has led our group to propose that overlapping systems may be involved (Lowery-Gionta et al., 2012; Sparrow et al., 2012; Thiele, 2012). Theoretically, CRF signaling is increased and NPY signaling is blunted when sufficient brain ethanol levels are achieved during binge-like drinking, stimulating continued excessive ethanol intake. Alterations in neuropeptide signaling are thought to initially be transient, but with repeated episodes of binge-like drinking these neuroplastic changes may become rigid, contributing to excessive dependence-like drinking.

One possibility is that procedures to promote binge-like ethanol drinking and procedures to induce dependence-like ethanol drinking are actually modeling the same phenotypes and trigger the same neuroplastic changes in the brain. Thus, it might be argued that the amount of ethanol exposure achieved with DID procedures is sufficient to induce a dependence-like state, and in fact that exposure to either DID procedures or ethanol vapor accomplish the same end-point. If this is true, one would predict that DID procedures should promote phenotypes consistent with ethanol dependence.

Therefore, the goal of the present project was to study the effects of a history of binge-like ethanol drinking on phenotypes that have previously been described following intermittent ethanol vapor exposure (Becker and Lopez, 2004; Crabbe et al., 1991; Kliethermes et al., 2004; Lopez et al., 2011; Philibin et al., 2012). A dependence-like state is typically associated with subsequent increases of voluntary ethanol consumption and self-administration (Becker and Lopez, 2004), and mice have been shown to exhibit elevated anxiety-like behaviors (Kliethermes et al., 2004), increased ataxia (Philibin et al., 2012), and increased sensitivity to

handling-induced convulsion (HICs) following 24-h or more of withdrawal after ethanol vapor exposure (Beadles-Bohling and Wiren, 2006; Homanics et al., 1998). Here, we assessed anxiety-like behaviors, ataxia, HIC, and finally voluntary ethanol consumption in mice with varying amounts of experience with binge-like ethanol drinking using DID procedures.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were 6-8 weeks of age and weighed 20-30 g upon arrival. Mice were housed individually in standard plastic cages and allowed to habituate to the environment for at least 1 week before experimental procedures were initiated. The animal colony room was maintained at ~22°C with a 12h/12h light/dark cycle (lights on at 0700 h). Mice had *ad libitum* access to food throughout all experiments and *ad libitum* access to water except during ethanol access, as noted below. All procedures used were in accordance with the National Institute of Health guidelines and were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Drinking-in-the-dark Procedure

All experiments utilized a 4 day drinking-in-the-dark (DID) procedure that our group and others have used previously to generate high levels of ethanol intake that are associated with blood ethanol concentrations (BECs) in excess of 80 mg/dl, typically greater than 100 mg/dl (Lowery-Gionta et al., 2012; Lowery et al., 2010; Rhodes et al., 2005; Rhodes et al., 2007; Sparrow et al., 2012). On days 1-3, beginning 3 h into the dark cycle, water bottles were removed from all cages and replaced with a bottle containing 20% (v/v) ethanol solution. Mice had 2 h of access to ethanol, after which the ethanol bottles were removed from cages and water bottles were replaced. The same procedure was followed on day 4 except that ethanol access was extended to 4 h. We used this schedule of ethanol access because Rhodes et al. (2005) noted robust

binge-like drinking with these procedures and because we have previously used this ethanol access schedule (Lowery-Gionta et al., 2012; Lyons et al., 2008; Sparrow et al., 2012; Sparta et al., 2008), thus allowing us to make direct comparisons of the present work with our previous research. Each 4 day DID procedure is referred to as a single binge-like ethanol drinking "cycle" or "episode" below. Between each binge-like drinking cycle, mice were given 3 days of rest with no access to ethanol. In Experiment 1 described below, mice were separated into groups balanced for body weights. Groups of mice experienced 1 (n = 13) or 6 (n = 13) binge-like drinking cycles and a third control group drank water throughout the binge-like drinking portion of the experiment (n = 13). In Experiment 2 described below, animals were divided into 5 groups balanced for body weights: The groups experienced 1, 3, 6, or 10 binge-like drinking cycles, and a fifth control group drank water only throughout the binge-like drinking portion of the experiment (n =10/group). For both experiments, initiation of binge-like drinking was staggered between groups such that all mice experienced their last binge-like drinking cycle at the same age. Though age of first ethanol exposure was different between groups (an unavoidable potential confound stemming from holding age of sacrifice constant between groups), it is important to note that all mice were of adult age at first ethanol exposure.

The EPM, open-field, rotarod, and HIC tests described below were all conducted in the animals' dark cycle to be consistent with the timing of DID procedures. The EPM and open-field tests were conducted in the dark; however, it was necessary for the rotarod and HIC tests to be conducted with light for data collection. Additionally, mice were transported to testing rooms on a cart covered with a tarp to limit light exposure.

Elevated Plus Maze (EPM): Assessment of Anxiety-Like Behavior

The EPM test is a pharmacologically validated model for the assessment of anxiety in rodents (Pellow et al., 1985). For both Experiments 1 and 2, the EPM test was initiated 24-h after the completion of the final binge-like drinking test. We have described the apparatus and the 5-min

procedure previously (Fee et al., 2004). The behavior in the EPM was recorded using a digital video camera with night vision capability, and measures were scored by condition-blinded individuals trained to identify the various dependent measures. With the EPM test, anxiety-like behavior is reflected as reduced number of entries into the open arms and in the time spent in the open arms, along with an increase in the number of entries and amount of time spent in the closed arms of the EPM.

Open-Field Locomotor Activity Test: Assessment of Anxiety-Like Behavior

The open-field test is commonly used to assess anxiety-like behavior in rodents, in which avoidance of the center portion of the open arena is thought to reflect heightened anxiety (Choleris et al., 2001; Fee et al., 2004). After the completion of the EPM test in Experiments 1 and 2, mice were transported in their home cages to an adjacent room and allowed to rest for 10 min. We have described the apparatus and procedure elsewhere (Fee et al., 2004). Testing sessions were 10 minutes in duration, and marginal time and distance traveled (cm), and central time and distance traveled (cm) in the open-field arena were measured over the course of the session.

Accelerating Rotarod Test: Assessment of Ataxia

Recent observations show that mice experiencing ethanol withdrawal exhibit elevated ataxia, and have difficulty maintaining balance on a rotarod apparatus (Philibin et al., 2012). After open-field testing in Experiment 2, mice were allowed to rest for 15 min in their home cage and were then placed on a rotarod apparatus (Ugo Basile, Italy) with an initial rotation speed of 4 rpm. The speed was gradually increased to 40 rpm over a period of 5 min and latency-to-fall and rpm at the time of fall were recorded. The test was repeated three more times, with 5 min between each test. Average latency-to-fall and rpm at time of fall were averaged over the 4 test trials.

Handling-Induced Convulsions (HIC): Assessment of Seizure Sensitivity

Thirty min after administration of the rotarod tests in Experiment 2, HIC tests were conducted. For HIC testing, each mouse was picked up (from its home cage) by the tail, and if this failed to elicit a convulsion, the mouse was spun gently through a 180^0 arc. The behavior of each mouse during the HIC test was video recorded and later scored independently by 2 condition-blinded raters (scores were averaged for analyses). The HIC rating scale ranged from 0-7, with a score of 0 given to mice showing no convulsions and a score of 7 given to mice exhibiting severe tonic-clonic convulsions. A detailed description of HIC procedures and the scoring scale is described elsewhere (Crabbe et al., 1991).

Continuous 2-Bottle Voluntary Ethanol Consumption

Approximately one week after the completion of binge-like ethanol drinking in Experiments 1 and 2, the mice were tested for voluntary ethanol consumption using a home cage 2-bottle choice procedure. Over 8 days, mice were given 24 h access to 2-bottles in their homecage, one containing tap water and the other containing a 10% (v/v) ethanol solution. The concentration of ethanol was changed every 8 days as follows: 10, 15, 20, 15, and 10% ethanol. The positions of the bottle were alternated every day to control for position preferences. Each drinking bottle was weighed every day to calculate fluid intake and body weights were recorded every 4 days.

Data Analyses

To obtain a measure that corrected for individual differences in body weight, grams of ethanol consumed per kilogram of body weight were calculated. Ethanol preference ratios were also calculated by dividing the volume of ethanol consumed by the total fluid (ethanol + water) consumption. For all experiments, differences between groups were analyzed using analysis of variance (ANOVA). With significant interaction effects, or main effects in the absence of significant interactions, post hoc comparisons were performed using Bonferroni corrected t-tests

to parse out group differences. Statistics were analyzed using SPSS (version 17.0; Armonk, NY) software. In all cases, $p<0.05$ (two tailed) was used to indicate statistical significance.

RESULTS

Drinking-in-the-dark

In Experiment 1, there were no significant differences between the groups that experienced 1 (5.42 ± 0.28 g/kg/4h) or 6 (6.05 ± 0.29 g/kg/4h) binge-like drinking cycles in terms of the amount of ethanol consumed during the final binge-like drinking session [$F_{(1,25)} = 2.486$, $p = 0.127$]. Similarly, in Experiment 2 there were no significant differences between the groups that experienced 1 (4.43 ± 0.29 g/kg/4h), 3 (5.09 ± 0.41 g/kg/4h), 6 (6.16 ± 0.36 g/kg/4h), or 10 (5.36 ± 0.31 g/kg/4h) binge-like drinking cycles in the amount of ethanol consumed during the final binge-like drinking session [$F_{(3,36)} = 1.374$, $p = 0.226$]. These data suggest that up to 10 binge-like drinking cycles did not significantly increase the level of binge-like ethanol drinking, which may reflect a ceiling effect stemming from the high levels of ethanol intake that are evident even after one DID cycle. Although we did not assess blood ethanol concentrations (BECs) in an attempt to avoid the potential confounding effects of stress on subsequent measures, the amount of ethanol consumed by mice on the final day of binge-like ethanol drinking was consistent with amounts that we have previously reported and which produced BECs of 80 mg/dL or greater (Lowery-Gionta et al., 2012; Lowery et al., 2010; Sparrow et al., 2012; Sparta et al., 2008).

Elevated Plus Maze (EPM): Assessment of Anxiety-Like Behavior

Data from the EPM tests for Experiments 1 and 2 are presented in Table 1. Data sets from both experiments failed to reveal any significant effects of binge-like ethanol drinking on subsequent anxiety-like behavior. In Experiment 1, relative to the water control group, mice that experienced 1 or 6 cycles of binge-like ethanol drinking exhibited no significant differences in open arm time

[$F_{(2,36)}$ = 0.146, p = 0.865] or percentage total time spent in open arms [$F_{(2,36)}$ = 0.145, p = 0.856]. Furthermore, there were no group differences in closed arm time [$F_{(2,37)}$ = 1.056, p = 0.358] or percentage of total time spent in closed arms [$F_{(2,37)}$ = 1.058, p = 0.357]. A lack of group differences in total arm entries [$F_{(2,37)}$ = 1.05, p= 0.360] suggests that the history of binge-like ethanol drinking did not impact overall activity (H_2O = 18.38 ± 1.20 entries; 1-cycle = 20.46 ± 1.07 entries; 6-cycle = 18.92 ± 0.84 entries).

In Experiment 2, relative to the water control group there was no evidence of alterations of anxiety-like behavior in mice that experienced 1 to 10 cycles of binge-like ethanol drinking. Thus, there were no group differences in open arm time [$F_{(4,44)}$= 2.012, p = 0.109] or percentage total time spent in open arms [$F_{(4,44)}$= 2.007, p = 0.110]. Furthermore, there were no group differences in closed arm time [$F_{(4,45)}$ = 2.016, p = 0.108] or percentage of total time spent in closed arms [$F_{(4,45)}$ = 2.013, p = 0.109]. Finally, there were no significant differences between groups on the number of total arm entries [$F_{(4,45)}$ = 0.448, p= 0.774], suggesting that the history of binge-like ethanol drinking did not impact overall activity (H_2O = 22.2 ± 1.10 entries; 1-cycle = 21.3 ± 1.59 entries; 3-cycle = 21.5 ± 1.68 entries; 6-cycle = 22.7 ± 1.32 entries; 10-cycle = 20.3 ± 1.03 entries).

Open-Field Locomotor Activity Test: Assessment of Anxiety-Like Behavior

Data from the open-field locomotor activity tests from Experiments 1 and 2 are presented in Table 2. As with the EPM test, data sets from both experiments failed to provide evidence of elevated anxiety-like behavior stemming from a history of binge-like ethanol drinking. For data from Experiment 1, there were no group differences in average distance traveled in the margins [$F_{(2,37)}$ = 0.545, p = 0.584], average time spent in margins [$F_{(2,37)}$ = 2.505, p = 0.095], average distance traveled in the center [$F_{(2,36)}$ = 0.867, p = 0.429], or average time spent in the center [$F_{(2,37)}$ = 0.507, p = 0.095]. Additionally, analysis of total distance traveled data failed to achieve statistical significance [$F_{(2,37)}$ = 0.688, p = 0.509], providing further support that a history of

binge-like ethanol drinking did not alter overall locomotor behavior (H_2O = 2982.38 ± 226.83 cm^2/10min; 1-cycle = 3278 ± 195.19 cm^2/10min; 6-cycle = 2956.57 ± 218.52 cm^2/10min; 10-cycle = 3146 ± 134.36 cm^2/10min).

The same pattern of results were observed from the open-field test of Experiment 2. There were no group differences in average distance traveled in the margins [$F_{(4,45)}$= 0.244, p = 0.912], average time spent in margins [$F_{(4,45)}$ = 0.112, p = 0.978], average distance traveled in the center [$F_{(4,45)}$ = 0.283, p = 0.887], and average time spent in the center [$F_{(4,45)}$ = 0.112, p = 0.978], and analysis of total distance traveled failed to achieve statistical significance [$F_{(4,45)}$ = 0.133, p = 0.969] (H_2O = 2942 ± 206.4 cm^2/10min; 1-cycle = 3094.5 ± 212.46 cm^2/10min; 3-cycle = 3037.7 ± 256.41 cm^2/10min; 6-cycle = 2989.7 ± 275.36 cm^2/10min; 10-cycle = 3146 ± 134.36 cm^2/10min).

Accelerating Rotarod Test: Assessment of Ataxia

Data averaged over the 4 rotarod tests performed in Experiment 2 are presented in Table 3. Separate one-way ANOVAs performed on latency to fall data [$F_{(4,45)}$ = 0.691, p = 0.602] and average RPM at fall data [$F_{(4,45)}$ = 0.724, p = 0.58] both failed to achieve statistical significance, indicating that a history of binge-like ethanol drinking did not promote increased ataxia.

Handling-Induced Convulsions (HIC): Assessment of Seizure Sensitivity

Data from the HIC test performed in Experiment 2 are presented in Table 3. An ANOVA performed on these data failed to achieve statistical significance [$F_{(4,45)}$ = 0.651, p = 0.629], suggesting that a history of binge-like ethanol drinking did not alter seizure sensitivity.

Continuous 2-Bottle Voluntary Ethanol Consumption

Data representing the average 8-day consumption of ethanol (g/kg) and average 8-day ethanol preference at each ethanol concentration tested in Experiment 1 (Fig. 1A and B, respectively) and Experiment 2 (Fig. 1C and D, respectively) are presented in Fig. 1. Separate two-way (ethanol concentration x number of binge-like drinking cycles) ANOVAs were performed to

analyze each data set. Analysis of ethanol consumption data from Experiment 1 revealed significant main effects of ethanol concentration [$F_{(4,148)}$ = 49.724, p < 0.001] and binge-like drinking cycles [$F_{(1,37)}$ = 10.279, p < 0.001], and a significant interaction effect [$F_{(8,148)}$ = 2.97, p = 0.004]. Post hoc Bonferroni corrected t-tests revealed that the group with a prior history of 6 binge-like ethanol drinking cycles voluntarily drank significantly more ethanol relative to the control group during access to 4 of the 5 ethanol solutions, and more than the 1-cycle group at 2 of the 5 concentrations (Fig. 1A). Analysis of ethanol preference ratio data from Experiment 1 showed significant main effects of ethanol concentration [$F_{(4,148)}$ = 58.905, p < 0.001] and binge-like drinking cycles [$F_{(1,37)}$ = 8.357, p = 0.001], but the interaction effect was not significant [$F_{(8,148)}$ = 1.923, p = 0.061]. A post hoc Bonferroni corrected t-test of the binge-like drinking cycles main effect revealed that only the 6-cycle binge-like ethanol drinking group preferred ethanol significantly more than the control group (Fig. 1B).

Analysis of the ethanol consumption data from Experiment 2 revealed significant main effects for ethanol concentration [$F_{(4,180)}$ = 65.420, p < 0.001] and binge-like drinking cycles [$F_{(4,45)}$ = 8.173, p < 0.001], and a significant interaction effect [$F_{(16,180)}$ = 2.076, p = 0.011]. Post hoc Bonferroni corrected t-tests revealed that relative to the control group, mice that had a prior history for 6 or 10 binge-like drinking cycles voluntarily drank significantly more ethanol during access to 4 of the 5 ethanol solutions. Mice with a history of 3 binge-like drinking cycles drank significantly more ethanol than the control group during access to 3 of the 5 ethanol solutions. Finally, mice with a history of 10 binge-like drinking cycles drank significantly more ethanol than the 1-cycle group at one concentration (Fig. 1C). Analysis of ethanol preference data from Experiment 2 showed significant main effects of ethanol concentration [$F_{(4,180)}$ = 59.915, p < 0.001] and binge-like drinking cycles [$F_{(4,45)}$ = 5.82, p = 0.001], but the interaction effect was not significant [$F_{(16,180)}$ = 0.984, p = 0.476]. A post hoc Bonferroni corrected t-test of the binge-like

drinking main effect showed that groups with a prior history of 3 to 10 binge-like ethanol drinking cycles showed significantly elevated ethanol preference relative to the control group (Fig. 1D).

DISCUSSION

Here we show that relative to mice with no prior history of binge-like ethanol drinking, a history of binge-like ethanol drinking using DID procedures in male C57BL/6J mice promoted subsequent increases of voluntary ethanol consumption and preference without altering anxiety-like behaviors (assessed by EPM and open-field locomotor activity tests), ataxia (assessed by the accelerating rotarod test), or sensitivity to HICs. While we cannot completely rule out the possibility that testing order could have impacted test outcomes (since test order was not counterbalanced), the lack of any trends suggests that this possibility is unlikely. Importantly, increased voluntary ethanol consumption and preference were greater in mice that experienced greater numbers of binge-like ethanol drinking cycles, with mice experiencing 6 or 10 binge-like drinking episodes showing the most robust increases of subsequent voluntary ethanol drinking and preference. Conversely, there was no instance in which mice with a prior history of just one binge-like drinking cycle exhibited a significant change from the control group in subsequent ethanol intake or preference. These observations are consistent with the hypothesis that alterations in the neurocircuitry that modulates binge-like ethanol drinking become more robust and longer-lasting with increasing numbers of binge-like drinking cycles. However, other phenotypes (i.e., anxiety-like behavior, ataxia, and HICs) that have been reported with procedures that induce a dependence-like state (i.e., ethanol vapor exposure) were not evident in mice experiencing up to 10 4-day cycles of binge-like ethanol drinking. Together, these observations make a strong case that DID and ethanol vapor procedures do not lead to the same end-point, and suggest that unlike ethanol vapor exposure procedures, excessive ethanol drinking stemming from DID procedures does not initially induce a dependence-like state.

However, the subsequent increases of voluntary ethanol consumption and preference that become more robust following repeated episodes of binge-like ethanol drinking may reflect the emergence of ethanol dependence, suggesting that DID procedures may be ideal for studying the early stages of the transition to ethanol dependence. We speculate that increased anxiety-like behavior, ataxia, and sensitivity to HIC may emerge with greater experience with binge-like ethanol drinking (i.e., more than 10 cycles of DID exposure).

The most striking results from the present data set were the robust and long-lasting increases of voluntary ethanol consumption and preference stemming from a history of binge-like ethanol drinking. These observations parallel those obtained with vapor inhalation models that have been shown to induce subsequent increases of voluntary self-administration or consumption of ethanol in rats (Funk et al., 2007; Gilpin et al., 2011) and mice (Becker and Lopez, 2004; Finn et al., 2007; Lopez et al., 2011). Importantly, increases of subsequent voluntary ethanol consumption in the present report resulted from animals voluntarily drinking excessive amounts of ethanol in a pattern and level that closely matches human binge drinking, rather than from forced exposure to ethanol vapor. Thus, as a model to study the neurobiology underlying the transition to an ethanol dependence-like state (Thiele, 2012), repeated binge-like drinking episodes using procedures such as DID arguably have greater face validity than models employing repeated intermittent ethanol vapor exposure.

Previous reports have shown that up to 24-h of withdrawal from ethanol vapor exposure is associated with elevated anxiety-like behaviors (Kliethermes et al., 2004), increased ataxia (Philibin et al., 2012), and increased sensitivity to HICs (Beadles-Bohling and Wiren, 2006; Becker and Veatch, 2002; Homanics et al., 1998) in mice. There are numerous possibilities that may explain differences between the present report and previous studies, including the amount, pattern, and route of ethanol exposure, the amount of ethanol withdrawal time before tests were administered, and the strain of mice used. For example, while HICs have been observed up to

24-h after ethanol withdrawal, HICs tend to be more robust in the first several hours after ethanol withdrawal (Beadles-Bohling and Wiren, 2006; Homanics et al., 1998), and strains other than C57BL/6J mice are more sensitive to withdrawal-induced HICs (Homanics et al., 1998). However, C57BL/6J mice have been reported to show elevated HICs 8-h after ethanol removal following 16-weeks of intermittent access to 20% ethanol in an escalation paradigm (Hwa et al., 2011), which is why we include this test in the present set of studies. Furthermore, strains other than C57BL/6J mice have been used to assess ataxia (Philibin et al., 2012) and anxiety-like behavior (Kliethermes et al., 2004) following ethanol vapor exposure.

Importantly though, our goal was to address the possibility that models of binge-like ethanol drinking and dependence-like ethanol drinking trigger the same phenotypes and associated neuroplasticity, a possibility that might seem particularly relevant given evidence of similar involvement of CRF and NPY signaling in binge-like and dependence-like ethanol drinking (Gilpin et al., 2011; Lowery-Gionta et al., 2012; Roberto et al., 2010; Sparrow et al., 2012). However, because we found that binge-like ethanol drinking did not promote many phenotypes thought to be hallmarks of ethanol dependence, we argue that the DID procedure models excessive ethanol intake prior to the onset of dependence. Consistent with this argument, we have found that a history of binge-like ethanol drinking blocks the ability of CRF to enhance GABAergic transmission in the central amygdala (CeA) of mice (Lowery-Gionta et al., 2012), while others have found that intermittent ethanol vapor exposure augments the ability of CRF to enhance GABAergic transmission in the CeA (Roberto et al., 2010). We would predict that more extensive experience with binge-like drinking episodes would lead to the development of neuroplastic alterations in line with models of ethanol dependence.

The possible mechanisms by which a history of binge-like ethanol drinking promotes subsequent increases of voluntary ethanol intake deserve consideration. It might be argued that mice previously exposed to DID procedures were more familiar with ethanol than the water

drinking control group, and thus more willing to consume ethanol during voluntary testing. However, this seems unlikely as the control mice would be expected to become familiar with ethanol during the early stages of voluntary consumption, yet group differences in voluntary drinking were evident throughout the 40-day test. Another possibility is that mice may have developed metabolic tolerance over the course of repeated DID episodes. While we did not measure BECs here, in two separate publications (Lowery-Gionta et al., 2012; Sparrow et al., 2012) we found that there were no significant differences between groups of mice experiencing 1 or 6 cycles of DID either in terms of the amount of ethanol that they consumed or the BECs that they achieved. Since similar BECs were achieved after similar levels of ethanol intake in mice with a history of 1 or 6 binge drinking cycles, metabolic tolerance does not appear to be a likely explanation for increased voluntary consumption of ethanol. The development of physiological tolerance is an attractive possibility, as repeated DID ethanol access was found to promote tolerance to the effects of ethanol injections on the balance beam test of ataxia (Linsenbardt et al., 2011). Additionally, a previous report identified low sensitivity to the aversive effects of ethanol (Holstein et al., 2011) as a potential mechanism that modulates binge-like ethanol drinking. Thus blunted sensitivity to the aversive effects of ethanol following binge-like drinking may promote subsequent increases of voluntary ethanol consumption.

At the level of neurocircuitry (Sprow and Thiele, 2012), CRF and NPY are two candidate pathways of particular interest. We have recently found that in mice with a 3-cycle history of binge-like ethanol drinking, CRF and NPY immunoreactivity and the effects of CRF or NPY on GABAergic transmission in the CeA were significantly altered for up to 24-h after ethanol removal (Lowery-Gionta et al., 2012; Sparrow et al., 2012). These observations suggest that binge-like ethanol drinking is associated with rigid neuroplastic alterations of CRF and NPY signaling in the CeA. We speculate that these changes contribute to the transition to ethanol dependence. Evidence of increased voluntary ethanol drinking resulting from a history of binge-

like ethanol drinking in the present report may reflect, in part, such changes in CRF and NPY signaling.

In summary, here we report that while repeated cycles of binge-like ethanol drinking promoted subsequent increases of voluntary ethanol intake, an effect that becomes more robust with increasing numbers of binge-like drinking episodes, a history of binge-like ethanol drinking did not impact other phenotypes thought to characterize a dependence-like state, including anxiety-like behavior, ataxia, and HICs. Together, these observations make a strong case that DID procedures (to model binge-like ethanol drinking) and ethanol vapor procedures (to model dependence-like drinking) do not lead to the same neurobiological end-point, at least initially. Unlike ethanol vapor exposure procedures, excessive ethanol drinking stemming from DID procedures does not initially induce a dependence-like state. However, the subsequent increases of voluntary ethanol consumption and preference that become more robust following repeated episodes of binge-like ethanol drinking may reflect the early stages of ethanol dependence, suggesting that DID procedures may be ideal for studying the transition to ethanol dependence.

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563 FIGURE CAPTION

564

565 **Fig. 1.** A history of repeated cycles of binge-like ethanol (20%, v/v) drinking significantly
566 augments subsequent 2-bottle choice voluntary ethanol consumption. Data points at each
567 concentration of ethanol (v/v) represent an 8-day average, and data are expressed as mean g of
568 ethanol consumed per kg of body weight per day (g/kg/day; A and C) or mean ethanol
569 preference (ethanol consumed / total fluid intake; B and D). The top row represents data
570 collected from Experiment 1 (A and B) and the bottom row represents data collected from
571 Experiment 2 (C and D). Data are expressed as mean ± SEM. Significance legend (based on
572 Bonferroni corrected t-tests): **a** = H_20 < 6-cycles; **b** = 1-cycle < 6-cycles; **c** = H_20 < 3-, 6-, and
573 10-cycles; **d** = H_20 < 6- and 10-cycles; **e** = 1-cycle < 10-cycles. Significant differences between
574 groups on the cycles main effect (indicated in B and D) are based on Bonferroni corrected t-
575 tests.

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TABLE 1: Average (Mean ± SEM) behavior in the elevated plus maze (EMP) tests from Experiments 1 and 2.

	Time in Open Arms (s)	Percentage of Time in Open Arms (%)	Time in Closed Arms (s)	Percentage of Time in Closed Arms (%)
Experiment 1				
H_20	35.8 ± 5.58	11.9 ±1.86	136.3 ± 10.67	45.4 ± 3.56
1-cycle	41.3 ± 7.10	13.76 ± 2.36	150.6 ± 9.23	49.2 ± 3.15
6-cycle	38.2 ± 7.95	12.74 ± 2.65	155.0 ± 8.66	51.7 ± 2.89
Experiment 2				
H_20	70.9 ± 10.0	23.6 ± 3.35	164.2 ± 14.13	54.7 ± 4.71
1-cycle	62.9 ± 12.86	21.0 ± 4.28	187.9 ± 16.13	62.7 ± 5.38
3-cycle	41.3 ± 5.78	13.8 ± 1.93	214.3 ± 10.0	71.4 ± 3.33
6-cycle	80.8 ± 9.79	26.9 ± 3.26	164.9 ± 11.12	55.0 ± 3.71
10-cycle	54.3 ± 12.67	18.1 ± 4.22	189.6 ± 19.58	63.2 ± 6.53

582 **Table 2: Average (Mean ± SEM) behavior in the open-field activity tests from**
583 **Experiments 1 and 2.**

	Marginal Distance (cm^2/10min)	Time in Margin (s)	Center Distance (cm^2/10min)	Time in Center (s)
Experiment 1				
H_20	2271.4 ± 198.88	505.3 ± 5.58	711.0 ± 36.61	94.7 ± 5.58
1-cycle	2501.5 ± 119.17	510.5 ± 7.59	673.8 ± 68.14	89.5 ± 7.59
6-cycle	2325.9 ± 155.85	521.3 ± 5.53	609.2 ± 60.66	78.7 ± 5.53
Experiment 2				
H_20	2194.7 ± 163.56	507.6 ± 9.60	747.2 ± 66.37	92.4 ± 9.60
1-cycle	2351.8 ± 162.53	509.1 ± 8.49	742.6 ± 71.58	90.9 ± 8.49
3-cycle	2418.1 ± 163.64	500.1 ± 14.81	655.3 ± 101.75	99.9 ± 14.81
6-cycle	2311.0 ± 277.12	505.0 ± 7.90	678.4 ± 56.81	95.0 ± 7.90
10-cycle	2414.6 ± 125.60	500.9 ± 16.04	732.1 ± 86.19	99.1 ± 16.04

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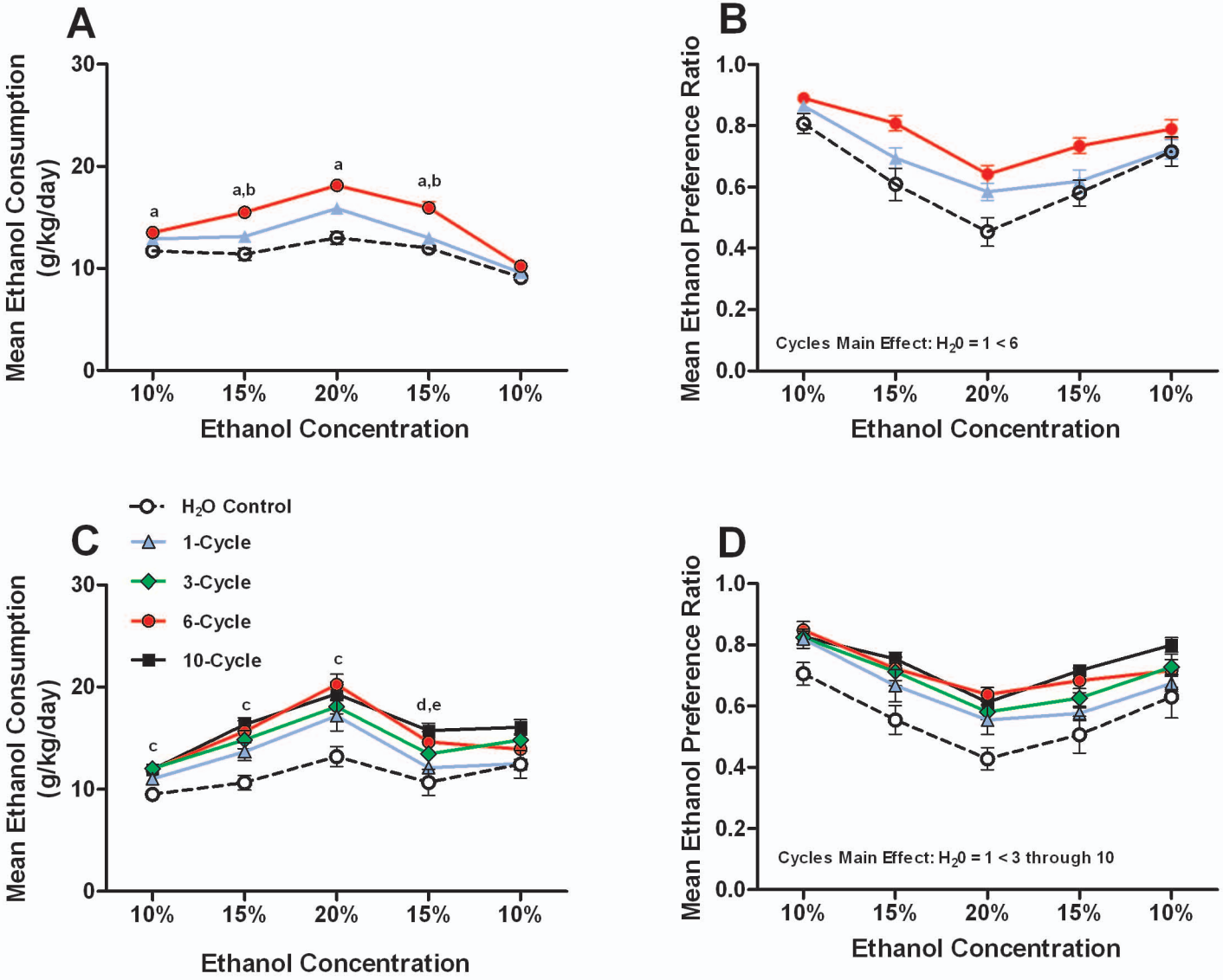
587 **Table 3: Average scores (mean ± SEM) from the rotarod and HIC test in Experiment 2.**

	Rotarod: Latency to Fall (s)	Rotarod: RPM at Fall	HIC Score
H_20	175.4 ± 13.12	25.9 ± 1.82	0.23 ± 0.12
1-cycle	168.0 ± 13.66	25.2 ± 1.96	0.14 ± 0.04
3-cycle	142.9 ± 18.00	25.5 ± 2.46	0.27 ± 0.07
6-cycle	151.8 ± 17.39	25.3 ± 2.54	0.18 ± 0.06
10-cycle	151.7 ± 17.23	26.6 ± 2.50	0.13 ± 0.06

588 HIC = Handling-induced convulsion (scored on a scale from 0 to 7).


A
Mean Ethanol Consumption (g/kg/day)
30
20
10
0
a
a,b
a
a,b
10%
15%
20%
15%
10%
Ethanol Concentration
B
Mean Ethanol Preference Ratio
1.0
0.8
0.6
0.4
0.2
0.0
Cycles Main Effect: H20 = 1 < 6
10%
15%
20%
15%
10%
Ethanol Concentration
H2O Control
1-Cycle
3-Cycle
6-Cycle
10-Cycle
C
Mean Ethanol Consumption (g/kg/day)
30
20
10
0
c
c
c
d,e
10%
15%
20%
15%
10%
Ethanol Concentration
D
Mean Ethanol Preference Ratio
1.0
0.8
0.6
0.4
0.2
0.0
Cycles Main Effect: H20 = 1 < 3 through 10
10%
15%
20%
15%
10%
Ethanol Concentration

The protective effects of the melanocortin receptor (MCR) agonist, melanotan-II (MTII), against binge-like ethanol drinking are facilitated by deletion of the MC3 receptor in mice

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Running Head: Melanocortin-3 Receptor and Binge-like Ethanol Drinking

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Abstract

Recent data have implicated the melanocortin (MC) system in modulating voluntary ethanol consumption. Administration of melanotan-II (MTII), a nonselective melanocortin receptor (MCR) agonist, reduces voluntary ethanol consumption in C57BL/6J mice. Previous studies have demonstrated that central infusion of MTII effectively reduced voluntary ethanol drinking in mutant mice lacking normal expression of MC3R ($MC3R^{-/-}$ mice) but failed to alter ethanol drinking in mice lacking expression of MC4R, demonstrating that central MTII administration reduces voluntary ethanol drinking by signaling through the MC4R. However, evidence shows that the neurocircuitry recruited during excessive binge-like ethanol drinking versus moderate ethanol drinking are not identical. Thus the present study sought to investigate the potential role of the MC3R in binge-like ethanol intake. To this end, the "drinking in the dark" (DID) procedure, a commonly used animal model of binge-like ethanol drinking, was employed. Wild-type $MC3R^{+/+}$ and $MC3R^{-/-}$ mice were given intracerebroventricular (i.c.v.) infusion of MTII (0.0, 0.25, 0.50, or 1.0 µg) before 4-hours of access to 20% (v/v) ethanol. Immediately after ethanol access, tail-blood samples were collected to assess blood ethanol concentrations (BECs). Consistent with previous findings, central administration of MTII blunted binge-like ethanol drinking in both $MC3R^{+/+}$ and $MC3R^{-/-}$ mice. Interestingly, all doses of MTII blunted binge-like ethanol drinking in $MC3R^{-/-}$ mice during the first hour of testing, while only the 1.0 µg dose reduced binge-like drinking in $MC3R^{+/+}$ mice. Thus, $MC3R^{-/-}$ mice were more sensitive to the protective effects of MTII. These data suggest that MC3Rs oppose the protective effects of MTII against binge-like ethanol drinking, and thus selective MC3R antagonists may have potential therapeutic roles in treating excessive ethanol drinking.

Keywords: Melanocortin, MC3 receptor, binge-like drinking, MTII, ethanol

Introduction

The melanocortin (MC) peptides, α-, β-, γ- melanocyte stimulating hormone (MSH), and adrenocorticotropic hormone (ACTH), are produced centrally by proopiomelanocortin (POMC)-expressing neurons within the arcuate nucleus of the hypothalamus, the nucleus of the solitary tract, and medulla (Jacobowitz and O'Donohue, 1978). These peptides, as well as their endogenous antagonists, agouti and agouti-related protein (AgRP), act through five seven-transmembrane G-protein coupled melanocortin receptor (MCR) subtypes. The MCRs within the rodent brain are predominantly comprised of the MC3R and MC4R subtypes, while MC1R, MC2R, and MC5R are expressed primarily in the periphery (Adan and Gispen, 1997; Barrett et al., 1994; Xia et al., 1995). Together, these ligands and their associated receptors, collectively referred to as the MC system, regulate a myriad of physiological functions including pigmentation (Robbins et al., 1993), sexual function (Argiolas et al., 2000), and appetite regulation (Fan et al., 1997; Giraudo et al., 1998), among others.

A growing body of literature has also implicated MCR signaling in modulating ethanol consumption. This association was first uncovered when it was reported that ethanol naïve alcohol preferring rats displayed abnormal expression of MC3R and MC4R within the nucleus accumbens and hypothalamus relative to their non-preferring counterparts (Lindblom et al., 2002). Extending on these findings, it was later demonstrated that central infusion of a nonselective MCR agonist, melanotan-II (MTII), attenuated voluntary ethanol consumption in these preferring rats (Ploj et al., 2002). Furthermore, central infusion of MTII reduced voluntary ethanol drinking in both wild-type and mutant mice deficient in MC3R, suggesting that the MC3R does not modulate voluntary drinking (Navarro et al., 2005). Navarro and colleagues also showed that a selective MC4R agonist blunted voluntary ethanol consumption in wild-type C57BL/6J mice, implicating the MC4R in modulating ethanol intake (Navarro et al., 2003).

Consistent with these findings, later work revealed that MTII had no effect on voluntary ethanol consumption in mutant mice lacking MC4R (Navarro et al., 2011). Taken together, these data suggest that central MTII reduces voluntary ethanol drinking by signaling through the MC4R, but not the MC3R.

However, the MC3R has been found to be expressed on POMC neurons (Bagnol et al., 1999), suggesting that this receptor may function as an autoreceptor. Subsequent *in vitro* investigations revealed that activation of these receptors using the selective MC3R agonist, $_{D}$-Trp^{8}-γ-MSH, induced a marked increase in IPSC frequency on POMC neurons (Cowley et al., 2001). Additionally, central infusions of this same compound caused a downregulation of POMC mRNA levels in rats (Lee et al., 2008). Further, rats receiving intracerebroventricular (i.c.v.) infusions of an MC3R agonist displayed increased food intake, while low doses of a MC3R antagonist reduced food intake. Together, these studies provide converging evidence that indicate the MC3R serves as an inhibitory autoreceptor on POMC neurons.

Recent converging evidence has suggested that different neurocircuitry modulates moderate level ethanol drinking versus excessive binge-like ethanol drinking (Sparta et al., 2008; Lowery et al., 2010; Lowery-Gionta et al., 2012). Despite the growing body of literature implicating the MC system in voluntary ethanol consumption, the role of the MC system in binge-like ethanol consumption remains relatively unexplored. We recently showed that mutant mice lacking AgRP exhibited blunted binge-like ethanol drinking, initial evidence that MCR signaling modulates binge-like drinking (Navarro et al., 2009). To further characterize the role of MC system in binge-like ethanol drinking, here we employed “drinking in the dark” (DID) procedures and used mutant mice lacking the MC3R (MC3R$^{-/-}$ mice) and their wild-type counterparts (MC3R$^{+/+}$ mice) to determine the potential contribution of the MC3R in modulating the protective effects of MTII against binge-like ethanol drinking. We found that consistent with

the previous data (Navarro et al., 2005)**,** infusions of MTII attenuated binge-like ethanol consumption in both $MC3R^{+/+}$ and $MC3R^{-/-}$ mice. However, we observed that MTII was more effective in reducing binge-like ethanol drinking in $MC3R^{-/-}$ relative to $MC3R^{+/+}$ mice. These data suggest that MC3Rs oppose the protective effects of MTII against binge-like ethanol drinking, and thus selective MC3R antagonists may have potential therapeutic roles in treating excessive ethanol drinking.

Materials and Methods

Animals

The generation of $MC3R^{-/-}$ has been described previously (Chen et al., 2000). Sixteen littermate knockout ($MC3R^{-/-}$) and ten wild-type ($MC3R^{+/+}$) mice maintained on a C57BL/6J background were bred in-house from heterozygous stock. Genotype was determined via polymerase chain reaction (PCR). All mice were housed in individual home cages located in a vivarium with an ambient temperature of approximately 22°C and a 12:12 h reverse light/dark cycle with lights off at 7:00am. Food and water were available *ad libitum* except where indicated below. It has previously been demonstrated that compounds targeting MCRs exhibit similar effects on ethanol intake in male and female mice (Navarro et al., 2005); therefore, both sexes were included in the present study in an effort to increase sample sizes. All procedures in this study were in compliance with the National Institute of Health guidelines, and all protocols were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Cannulation Surgery and Infusion Procedure

Prior to testing, mice underwent cannulation surgery targeting the left lateral ventricle, which has been described previously (Navarro et al., 2003, 2005, 2011). Following surgery, mice were given approximately a week to recover before testing. Following completion of testing, cannula placement was verified histologically. The non-selective MCR agonist

melanotan-II (MTII; American Peptide Company, Sunnyvale, CA) was dissolved in 0.9% saline to reach the desired concentration (0.0, 0.25, 0.5, or 1.0 µg). All doses were infused in a 1.0 µl volume using a Hamilton syringe (Hamilton Company USA, Reno, NV), which was administered manually over the course of one minute. This injector was left in place for an extra 30 seconds to allow for diffusion and to prevent reflux of the compound up the cannula tract. Following the infusion, mice were returned to their homecages.

"Drinking in the Dark" Procedures

A four day DID procedure was used to model binge-like ethanol drinking (Rhodes et al., 2005). It has previously been shown that mice in this paradigm are able to achieve a blood ethanol concentrations (BECs) that surpass the 80 mg/dl criterion used by the National Institute on Alcohol Abuse and Alcoholism to define an episode of binge drinking (National Institute on Alcohol Abuse and Alcoholism, 2004; Rhodes et al., 2007) and that drinking using this method is likely not motivated by caloric need (Lyons et al., 2008). For the first three days of DID, $MC3R^{+/+}$ and $MC3R^{-/-}$ mice were weighed and given mock infusions at the beginning of the dark cycle in order to acclimate the animals to the infusion procedure. Water bottles were removed three hours into the dark cycle and replaced with a single bottle of ethanol (20% v/v) for two hours. Water bottles were returned to the mice at the end of each day of testing. Treatment groups were equated based on ethanol consumption on days 1-3. On the fourth day of DID, mice were infused with a 0.0, 0.25, 0.5 or 1.0 µg dose of MTII as described above. These doses of MTII have been determined previously to be effective in modulating ethanol intake (Navarro et al., 2011). Binge-like ethanol consumption was assessed on this fourth day of testing, which followed the same schedule as the first three days with the exception that ethanol access was extended to four hours. Consumption measures were collected at the first hour of ethanol access as well as at the end of the four hours of testing in order to measure the immediate and

prolonged effects of the drug on ethanol intake, respectively. Tail bloods were collected from each animal at the end of the 4-hour testing period in order to determine BECs using an alcohol analyzer (Analox Instruments, Lunenburg, MA). A Latin square design was utilized such that the animals received each dose (vehicle, 0.25, 0.5, and 1.0 µg of MTII) in a counterbalanced order. Mice were given at least three days from testing between subsequent DID sessions in order to avoid carryover effects of the drug.

Data Analysis

Given the small sample size and variability between group variance, nonparametric tests of significance were used for statistical analyses (Siegel, 1956). The effect of dose (vehicle, 0.25, 0.5, or 1.0 µg) of MTII on binge-like ethanol consumption and BEC were assessed using the nonparametric Friedman Tests conducted on each genotype ($MC3R^{-/-}$ and $MC4R^{+/+}$ mice). One animal's tail blood sample was lost during centrifugation and was eliminated from BEC analysis. When omnibus Friedman Tests yielded significant results, planned comparisons were performed using nonparametric Wilcoxon signed-rank tests to assess the effect of each dose of MTII (0.25, 0.5, or 1.0 µg) versus vehicle within each genotype. Bonferroni's adjustments were made to account for multiple comparisons ($\alpha = 0.0167$). Additionally, a Pearson correlation was performed to examine the relationship between ethanol consumed over the four test and BECs collected immediately after ethanol testing.

Results

Friedman's test revealed that, relative to vehicle, central infusions of MTII significantly reduced binge-like ethanol consumption during the first hour of testing among both $MC3R^{+/+}$ and $MC3R^{-/-}$ mice ($\chi^2_{(3)} = 9.034$, $p = 0.029$; $\chi^2_{(3)} = 12.273$, $p = 0.007$, respectively; Fig. 1A). Further investigation using the Wilcoxon signed-rank test revealed that, among $MC3R^{-/-}$ mice, all doses (0.25, 0.5, and 1.0 µg) of MTII significantly reduced ethanol intake relative to vehicle during the

first hour (Z = -2.449, p = 0.007; Z = -2.982, p = 0.002 and Z = -2.691, p = 0.004, respectively). However, among $MC3R^{+/+}$ mice, only the highest dose (1.0 µg) of MTII was effective at reducing ethanol consumption (Z = -2.524, p = 0.006). MTII infusion resulted in a marked reduction in binge-like ethanol consumption in $MC3R^{-/-}$, but not $MC3R^{+/+}$ mice at the end of the four hour binge episode ($\chi^2_{(3)}$ = 15.153, p = 0.002; $\chi^2_{(3)}$ = 4.680, p = 0.197, respectively; Fig. 1B). Further examination using Wilcoxon signed-rank tests performed on 4-hour binge-like ethanol consumption data from $MC3R^{-/-}$ mice revealed that only the highest dose of MTII was able to significantly blunt ethanol consumption relative to the vehicle condition (Z = -2.726, p = 0.003).

Omnibus analysis of differences in BECs using the Friedman Tests revealed no significant effect of MTII on BEC for either genotype ($MC3R^{-/-}$: $\chi^2_{(3)}$ = 2.354, p = 0.502; $MC3R^{+/+}$: $\chi^2_{(3)}$ = 2.969, p = 0.396; Fig. 1C). Although these analyses revealed no significant differences in BEC as a result of drug treatment, there was a strong, positive relationship between BEC and ethanol consumed over the four hour period for both genotypes ($MC3R^{-/-}$: $r_{(57)}$ = 0.596, p < 0.001; $MC3R^{+/+}$: $r_{(38)}$ = 0.631, p < 0.001).

Discussion

These studies examined the central actions of the nonselective MCR agonist MTII, on binge-like ethanol drinking in $MC3R^{-/-}$ and wild-type $MC3R^{+/+}$ mice. Overall, the present results confirmed previous findings showing that centrally administered MTII attenuates ethanol consumption (Navarro et al., 2003, 2005, 2011). The observations made here add to the literature by showing that central administration of MTII also results in a decrease in binge-like ethanol intake in both $MC3R^{-/-}$ and $MC3R^{+/+}$ mice.

Importantly, we observed that mice with normal expression of MC3R were less sensitive to the protective effects of MTII against binge-like ethanol drinking relative to mice deficient of MC3R. This report, to the best of our knowledge, provides the first evidence that the MC3R

modulates the actions of centrally administered MTII on binge-like ethanol drinking. An interesting explanation for this phenomenon is that the MC3R acts as an inhibitory autoreceptor on POMC neurons. Thus, in the absence of the MC3R, both MTII and endogenous α-MSH participate in tandem to reduce binge-like ethanol drinking, while activation of the MC3R by MTII in wild-type mice inhibits the release of endogenous α-MSH. Previous studies have already identified MC3R as being expressed on POMC neurons (Bagnol et al., 1999). What is more, data from electrophysiological, immunohistochemistry, and behavioral studies report decreased activity in POMC neurons following selective activation of MC3Rs (Cowley et al., 2001; Lee et al., 2008). Although AgRP neurons within the arcuate nucleus express MC3R (Bagnol et al., 1999), it has previously been demonstrated that central administrations of a selective MC3R agonist suppresses levels of POMC mRNA expression but has no effect on AgRP gene expression (Lee et al., 2008). Together, these studies reinforce the idea that the MC3R acts as an inhibitory autoreceptor on the POMC circuit, most likely by inhibiting transmission of α-MSH from POMC neurons. A second possibility is that the MC3R, in some circuits, exists as a postsynaptic receptor and functionally opposes the downstream actions of MC4R signaling. The observation that genetic deletion of MC3R produces a mouse that is more susceptible to the attenuating effects of MTII on binge-like ethanol drinking is consistent with either a presynaptic autoreceptor or a postsynaptic receptor that functionally opposes the actions of the MC4R.

The fact that no differences were found in BECs between vehicle-treated and MTII-treated mice may be related to the time course of each animal's drinking. Specifically, tail blood samples were collected only at the end of the 4-hour test. As such, drinking that occurs toward the beginning of the test session may be less influential on the measured BEC than drinking that occurs more towards the end of the session. This is especially likely considering the rather rapid

decline of BEC that is characteristic of mice (Livey et al., 2003). As the protective effects of MTII on ethanol intake were most evident at the beginning of the 4-hour test, it seems likely that increased consumption later in the four hour test session may have masked the effects of MTII treatment on BECs. Importantly, although no differences were found in BECs between treatment conditions, there was a strong, direct relationship between the amount ethanol consumed and the measured BECs for both genotypes.

It may be surprising that the observations made here differ from previous studies examining the role of the MC3R in ethanol drinking, which found no significant contribution of the MC3R on ethanol drinking (Navarro, et al., 2005). However, two key differences exist that may account for this. First, the previous study measured 24-hour voluntary ethanol consumption procedure while the present experiment utilized the limited access DID procedure to model binge-like ethanol consumption. Thus, it is possible that MC3Rs are involved in the modulation of excessive binge-like ethanol drinking but not with more moderate levels of ethanol intake that are characteristic of voluntary ethanol consumption procedures. More importantly, our study used multiple doses (0.25, 0.5 and 1.0 μg) of MTII while only one dose (1.0 μg) was used previously. Even in the current report, we may have missed genotype differences in MTII sensitivity as the highest dose of MTII blunted binge-like ethanol drinking in both genotypes, at least at the 1-hour measure (Fig. 1A). It is not until all of the doses of MTII are taken into account that a pattern emerges that indicates loss of the MC3R augments the protective effects of MTII.

Given the involvement of the MC system in feeding behavior, these data further underscore the overlapping pathways involved in alcohol and food consumption. Indeed, it has previously been demonstrated that, in addition to suppressing alcohol consumption, treatment with centrally-administered MTII in doses used here (0.5 or 1.0 μg) also reduced the

consumption of food, sucrose solution (caloric reinforcers) and saccharin solution (a salient non-caloric reinforcer), but not water drinking (Navarro et al., 2005; 2011). These observations are consistent with the idea that the central MC system modulates the consumption of natural reinforcers (food and sweet solutions) and ethanol, regardless of caloric content. Moreover, a wealth of neurochemical systems involved in both alcohol and food consumption have already been identified (see Thiele et al., 2003 for review). A multitude of clinical reports have described a relatively high rate of co-morbidity between alcohol abuse and eating disorders reported (Dansky et al., 2000; Higuchi et al., 1993; Holderness et al., 1994), which is not surprising considering both conditions involve aberrant consummatory behavior and a loss of control. Moreover, in patients presenting both alcoholism and eating disorders, it is often found that successful treatment of one disorder also proves to be efficacious to both conditions (Daniels et al., 1999; Dawe and Staiger, 1998). Given these circumstances, examination of therapeutic drugs that act on these shared neurochemical systems, such as the melanocortin system, may prove to be a worthwhile endeavor.

In summary, we have demonstrated a possible role for the MC3R in modulating binge-like ethanol consumption. Specifically, the MC3R appears oppose the protective effects of MTII against binge-like ethanol drinking, which may indicate that the MC3R serves as an autoreceptor that prevents the release of endogenous α-MSH, or as a postsynaptic receptor that functionally opposes the downstream actions of the MC4R. It will be important for future studies to further characterize the contribution of the MC3R in modulating ethanol consumption. The current observations suggest that MC3R antagonists may have therapeutic value in combating alcohol abuse disorders, and may augment the ability of MC4R agonists to protect against binge drinking.

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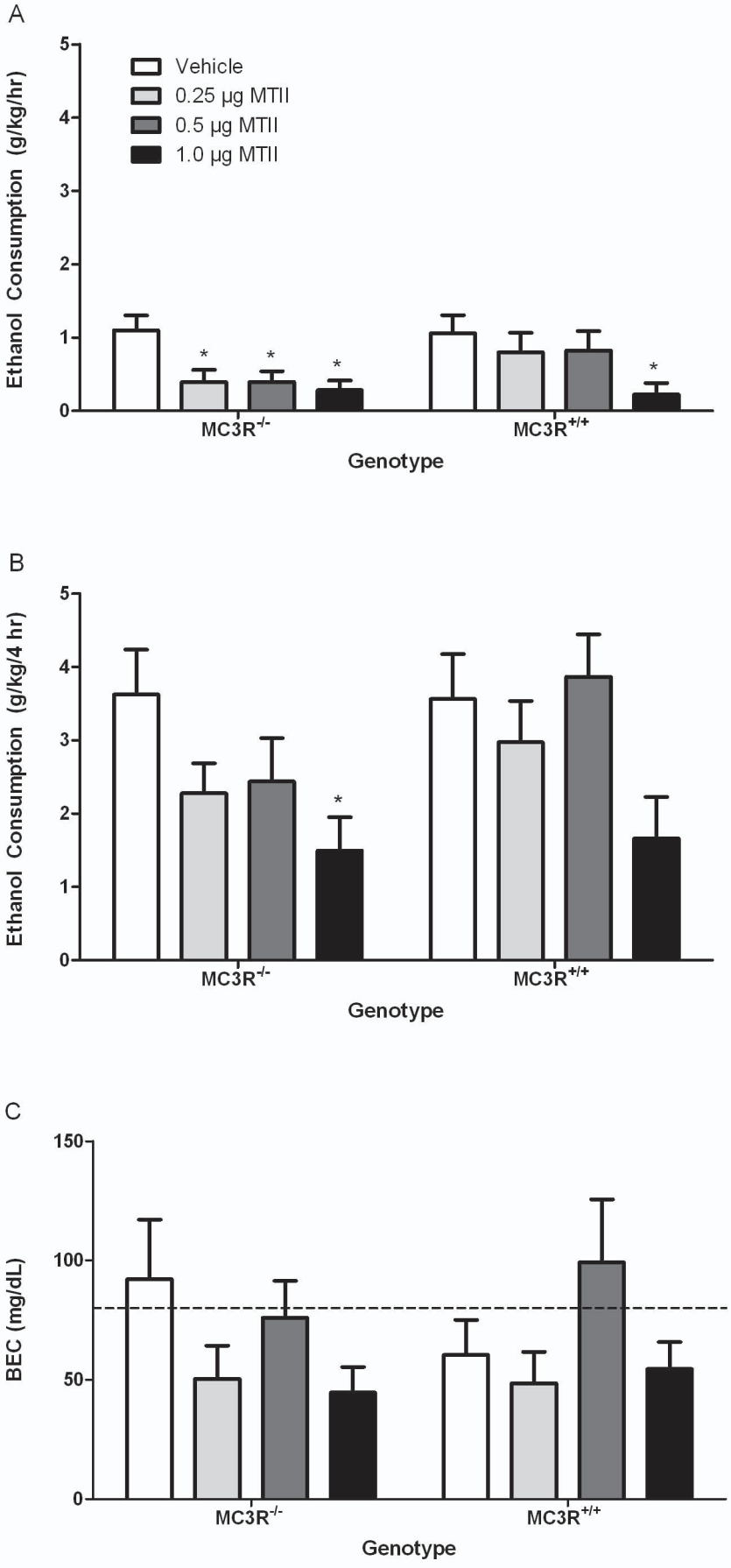
Figure Caption

Figure 1. Ethanol consumed (g/kg) among $MC3R^{-/-}$ and $MC3R^{+/+}$ mice during the first hour (A), and total four hours (B) of the binge-like ethanol consumption, as well as BEC (mg/dl) measured immediately following testing (C). During the first hour, $MC3R^{-/-}$ mice displayed a reduction in binge-like ethanol drinking at all doses tested. Alternatively, significant reductions in drinking were only observed following treatment with the highest dose (1.0 µg) of MTII in $MC3^{+/+}$ mice. Similarly, at the end of the 4-hour test, only the highest dose of MTII caused a reduction in binge-like ethanol drinking in $MC3R^{-/-}$ mice. No significant effect of MTII was observed on BEC regardless of genotype. Data are presented as mean $\pm$ SEM. * signifies $p < .05$ relative to vehicle within that genotype. Dashed line in (C) denotes 80 mg/dl BEC.


A
Vehicle
0.25 µg MTII
0.5 µg MTII
1.0 µg MTII
Ethanol Consumption (g/kg/hr)
5
4
3
2
1
0
*
*
*
*
MC3R-/-
MC3R+/+
Genotype
B
Ethanol Consumption (g/kg/4 hr)
5
4
3
2
1
0
*
MC3R-/-
MC3R+/+
Genotype
C
BEC (mg/dL)
150
100
50
0
MC3R-/-
MC3R+/+
Genotype

Assessment of Voluntary Ethanol Consumption and the Effects of a Melanocortin (MC) Receptor Agonist on Ethanol Intake in Mutant C57BL/6J Mice Lacking the MC-4 Receptor

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Background: The melanocortin (MC) system is composed of peptides that are cleaved from the polypeptide precursor proopiomelanocortin (POMC). Recent evidence shows that chronic exposure to ethanol significantly blunts central MC peptide immunoreactivity and MC receptor (MCR) agonists protect against high ethanol intake characteristic of C57BL/6J mice. Here, we assessed the role of the MC-4 receptor (MC4R) in voluntary ethanol intake and in modulating the effects of the nonselective MCR agonist melanotan-II (MTII) on ethanol consumption.

Methods: To assess the role of the MC4R, MC4R knockout ($Mc4r^{-/-}$) and littermate wild-type ($Mc4r^{+/+}$) mice on a C57BL/6J background were used. Voluntary ethanol (3, 5, 8, 10, 15, and 20%, v/v) and water intake were assessed using standard two-bottle procedures. In separate experiments, $Mc4r^{-/-}$ and $Mc4r^{+/+}$ mice were given intracerebroventricular (i.c.v.) infusion of MTII (0, 0.5, or 1.0 μg/1 μl) or intraperitoneal (i.p.) injection of MTII (0 or 5 mg/kg/5 ml). The effects of MTII (0 or 0.5 μg/1 μl, i.c.v.) on 10% sucrose and 0.15% saccharin intake were assessed in C57BL/6J mice.

Results: $Mc4r^{-/-}$ mice showed normal consumption of ethanol over all concentrations tested. I.c.v. infusion of MTII significantly reduced ethanol drinking in $Mc4r^{+/+}$ mice, but failed to influence ethanol intake in $Mc4r^{-/-}$ mice. When administered in an i.p. injection, MTII significantly reduced ethanol drinking in both $Mc4r^{-/-}$ and $Mc4r^{+/+}$ mice. MTII attenuated consumption of caloric (ethanol, sucrose, and food) and noncaloric (saccharin) reinforcers.

Conclusions: When given centrally, the MCR agonist MTII reduced ethanol drinking by signaling through the MC4R. On the other hand, MTII-induced reduction of ethanol drinking did not require the MC4R when administered peripherally. Together, the present observations show that the MC4R is necessary for the central actions of MCR agonists on ethanol drinking and that MTII blunts the consumption natural reinforcers, regardless of caloric content, in addition to ethanol.

Key Words: Ethanol Consumption, Melanocortin, MC-3 Receptor, MC-4 Receptor, C57BL/6J, Food Intake.

THE MELANOCORTIN (MC) SYSTEM is composed of peptides that are cleaved from the polypeptide precursor proopiomelanocortin (POMC). Central MC peptides are produced by neurons within the hypothalamic arcuate nucleus, the nucleus of the solitary tract, and the medulla (Crine et al., 1978; Dores et al., 1986; Hadley and Haskell-Luevano, 1999; Jacobowitz and O'Donohue, 1978; O'Donohue and Dorsa, 1982) and include adrenocorticotropic hormone, α-melanocyte-stimulating hormone (α-MSH), β-MSH, and γ-MSH (Hadley and Haskell-Luevano, 1999). Because of a lack of critical dibasic site, β-MSH is not processed in rodent brain (Pritchard et al., 2002). MC neuropeptides act through at least five receptor subtypes, namely MC-1 receptor (MC1R), MC2R, MC3R, MC4R, and MC5R, all of which couple to heterotrimeric Gs-proteins that stimulate adenylyl cyclase activity (Hadley and Haskell-Luevano, 1999). MC receptors (MCRs) in the rodent brain are primarily comprised of the MC3R and MC4R subtypes (Adan and Gispen, 1997), whereas MC1R and MC5R are detected at low levels and only in limited brain regions while the MC2R is expressed primarily in the adrenal cortex (Adan and Gispen, 1997; Barrett et al., 1994; Xia et al., 1995).

It is well established that MCR signaling is involved in the regulation of appetite and energy homeostasis (Gao and Horvath, 2008). A growing literature suggests that there is

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overlapping peptide control of ethanol consumption and feeding behavior (Thiele et al., 2003, 2004), which includes recent evidence that MCR signaling modulates neurobiological responses to ethanol. MCR and α-MSH expression have been identified in brain regions that modulate the reinforcing properties of ethanol, including the nucleus accumbens (NAc), ventral tegmental area, the bed nucleus of the stria terminalis, and amygdala (Bloch et al., 1979; Dube et al., 1978; Jacobowitz and O'Donohue, 1978; O'Donohue and Jacobowitz, 1980; O'Donohue et al., 1979; Yamazoe et al., 1984). Genetic evidence and pharmacological evidence implicate the MC system in the control of voluntary ethanol consumption. Relative to ANA (Alko, Nonalcohol) rats, AA (Alko, Ethanol) rats, selectively bred for high ethanol intake, have significantly lower levels of MC3R in the shell of the NAc, and significantly higher levels of MC3R in the paraventricular, arcuate, and ventromedial nuclei of the hypothalamus. AA rats also have high levels of MC4R in the ventromedial nucleus of the hypothalamus (Lindblom et al., 2002). These data suggest that the high ethanol drinking by AA rats may be mediated, in part, by alterations in central MCR signaling. Consistent with this hypothesis, intracerebroventricular (i.c.v.) infusion of the potent nonselective MCR agonist melanotan-II (MTII) significantly reduced voluntary ethanol drinking by AA rats (Ploj et al., 2002). Similarly, we have found that i.c.v. infusion of MTII and a selective MC4R agonist reduced ethanol drinking (Navarro et al., 2003, 2005), while ventricular infusion of the nonselective MCR antagonist agouti-related protein (AgRP) significantly increased ethanol drinking (Navarro et al., 2005), by high-ethanol-drinking C57BL/6J mice. Consistent with pharmacological data, genetic deletion of endogenous AgRP reduced ethanol-reinforced lever pressing and binge-like ethanol drinking in C57BL/6J (Navarro et al., 2009). Ethanol also has direct effects of central MC and AgRP activity. Thus, chronic exposure to ethanol significantly reduced α-MSH immunoreactivity in specific regions of the rat brain (Navarro et al., 2008), and acute administration of ethanol significantly increased AgRP immunoreactivity in the arcuate nucleus of the hypothalamus of C57BL/6J mice (Cubero et al., 2010).

The MCRs that modulate neurobiological responses to ethanol remain unclear. With respect to ethanol consumption, we found that MTII was similarly effective at reducing ethanol intake in both MC3R knock-out ($Mc3r^{-/-}$) and littermate wild-type ($Mc3r^{+/+}$) mice (Navarro et al., 2005). Furthermore, i.c.v. infusion of the highly selective MC4R agonist, cyclo(NH-CH_2-CH_2-CO-His-D-Phe-Arg-Trp-Glu)-NH_2, dose-dependently reduced ethanol drinking by C57BL/6J mice (Navarro et al., 2005). These data suggest that the MC3R does not modulate MCR agonist-induced reductions of ethanol consumption and that the MC4R is a likely candidate. The first goal of the present report was to directly assess the role of the MC4R. To this end, we examined voluntary ethanol consumption and the effects of centrally and peripherally administered MTII on ethanol intake, in $Mc4r^{-/-}$ and littermate $Mc4r^{+/+}$ mice. The second goal was to further characterize the effects of MTII on consumption of other caloric (food and sucrose) and noncaloric (saccharin) reinforcers.

MATERIALS AND METHODS

Animals

The generation of $Mc4r^{-/-}$ mice has been described elsewhere (Huszar et al., 1997). The $Mc4r^{-/-}$ mice were originally derived on a mixed 129/SvJ × C57BL/6J genetic background and show increased body weight and feeding behavior beginning at about 3-4 months of age (Huszar et al., 1997; Ste Marie et al., 2000). For the present work, we backcrossed $Mc4r^{-/-}$ mice to a C57BL/6J genetic background for 8 generations. Despite the lack of the MC4R, $Mc4r^{-/-}$ mice show normal brain expression of MC3R mRNA (Rowland et al., 2010). Littermate knockout and wild-type mice were used, and approximately equal numbers of male and female mice were used in each treatment condition. Because we have previously found no sex differences in the effect of MTII on ethanol consumption in C57BL/6J mice (Navarro et al., 2005), and because of low numbers of male and female mice within each treatment condition, sex was not included as a factor in analyses described later. The genetic status of all mice was determined using polymerase chain reaction (PCR) procedures, and mice were approximately 6 weeks of age at the beginning of experiments. We also used male C57BL/6J mice that were purchased at 6 weeks of age from Jackson Laboratory (Bar Harbor, ME). Mice were individually housed in polypropylene cages with corncob bedding and had ad libitum access to water and standard rodent chow (Tekland, Madison, WI) throughout each experiment. The colony room was maintained at approximately 22°C with a reverse 12:12 hours light:dark cycle with lights off at 10:00 AM. All procedures used in this study were in compliance with the National Institute of Health guidelines, and all protocols were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Experiment 1: Two-Bottle Consumption of Ethanol, Sucrose, Saccharin, and Water

$Mc4r^{-/-}$ ($n = 8$) and $Mc4r^{+/+}$ ($n = 9$) mice were tested for voluntary ethanol consumption using a homecage 2-bottle choice procedure. Over 4 days, mice were given 24-hour access to 2-bottles on their homecage, one containing tap water and the other containing a 3% (v/v) ethanol solution. The concentrations of ethanol were then increased to 5, 8, 10, 15, and 20% every 4 days. The positions of the bottles were alternated every 2 days to control for position preferences. Each drinking bottle was weighed every 2 days, and body weights were recorded every 4 days. An empty cage was used for the placement of dummy bottles (1 ethanol and 1 water), and fluid lost from each of these bottles was subtracted off the consumption totals as a control for fluid spillage. A separate set of $Mc4r^{-/-}$ ($n = 12$) and $Mc4r^{+/+}$ ($n = 12$) mice were tested for voluntary consumption of 0.15% (w/v) saccharin solution versus water followed by 10% (w/v) sucrose solution versus water in a two-bottle test. Mice were given access to each sweet solution for 2 days.

Experiment 2: Ethanol Consumption Following Central Infusion of the MCR Agonist MTII

Mice were anesthetized with a cocktail of ketamine (117 mg/kg) and xylazine (7.92 mg/kg) and surgically implanted with a 26-gauge guide cannula (Plastic One, Roanoke, VA) aimed at the left lateral ventricle, with the following stereotaxic coordinates: 0.2 mm posterior to bregma, 1.0 mm lateral to the midline, and 2.3 mm ventral to the surface. Mice were allowed to recover for approximately 2 weeks before experimental procedures were initiated. After experimental

procedures, cannula placement was verified histologically. I.c.v. infusions were given in a 1.0 μl volume over a 1-minute period using a 33-gauge injector needle that extended 0.5 mm beyond that guide cannula. Compounds were administered manually with a 1-μl Hamilton syringe. The injectors were left in place for an additional 1 minute to allow for drug diffusion and to minimize vertical capillary action along the injector tract when it was removed.

After recovery from surgery, animals received 5 days of habituation to 24 hour, 2-bottle consumption with one bottle containing water and the second bottle containing a solution of 10% (v/v) ethanol. Following habituation to 2-bottle drinking, mice from each genotype were distributed into groups that were equated based on ethanol consumption measured during the last 3 days of baseline. On the test day, mice were weighed, and ethanol, water, and food were removed from their cages 2 hours before the beginning of the dark cycle. Approximately 30- to 15-minutes before the beginning of the dark cycle, mice were given i.c.v. infusions of a 0.5 ($Mc4r^{-/-}$ mice, $n = 11$; $Mc4r^{+/+}$ mice, $n = 8$)- or 1 ($Mc4r^{-/-}$ mice, $n = 6$; $Mc4r^{+/+}$ mice, $n = 5$)-μg dose of MTII (Bachem, Torrance, CA) dissolved 0.9% saline, or an equal volume of 0.9% saline ($Mc4r^{-/-}$ mice, $n = 8$; $Mc4r^{+/+}$ mice, $n = 7$). We have previously found that the 1-μg dose of MTII was effective in reducing ethanol intake in C57BL/6J mice (Navarro et al., 2003, 2005). We chose MTII as we previously assessed the effects of MTII in $Mc3r^{-/-}$ mice (Navarro et al., 2005) and could thus make direct comparisons between studies. The 10% ethanol solution, water, and food were returned immediately before the dark cycle. Intake measures were recorded 6 hours later.

Experiment 3: Ethanol Consumption Following Peripheral Administration of the MCR Agonist MTII

$Mc4r^{-/-}$ and $Mc4r^{+/+}$ mice received 5 days of habituation to 24 hour, 2-bottle consumption with 1 bottle containing water and the second bottle containing a solution of 10% (v/v) ethanol. Following habituation to 2-bottle drinking, mice from each genotype were distributed into groups that were equated based on ethanol consumption measured during the last 3 days of baseline. On the test day, mice were weighed, and ethanol, water, and food were removed from their cages 2 hours before the beginning of the dark cycle. Approximately 30- to 15-minutes before the beginning of the dark cycle, mice were given an intraperitoneal (i.p.) injection of a 5 mg/kg dose of MTII dissolved in 0.9% saline ($Mc4r^{-/-}$ mice, $n = 15$; $Mc4r^{+/+}$ mice, $n = 14$) or an equal volume of 0.9% saline given in a 5 ml/kg volume ($Mc4r^{-/-}$ mice, $n = 15$; $Mc4r^{+/+}$ mice, $n = 15$). We chose the 5 mg/kg dose of MTII because it falls between doses (2 and 10 mg/kg) that have been shown to effectively attenuate feeding behavior (Chen et al., 2000; Choi et al., 2003). The 10% ethanol solution, water, and food were returned immediately before the dark cycle. Intake measures were recorded 6 hours later.

Experiments 4 and 5: Sucrose and Saccharin Solution Consumption Following Central Infusion of the MCR Agonist MTII

Surgery for cannula placement and i.c.v. infusion procedures were the same as described in Experiment 2. After recovery from surgery, animals received 5 days of habituation to 24 hour, 2-bottle consumption with one bottle containing water and the second bottle containing a solution of 10% (w/v) sucrose (Experiment 4) or 0.15% (w/v) saccharin (Experiment 5). Following habituation to 2-bottle drinking, mice from each genotype were distributed into groups that were equated based on sweet solution consumption measured during the last 3 days of baseline. On the test day, mice were weighed, and sweet solution, water, and food were removed from their cages 2 hours before the beginning of the dark cycle. Approximately 30 to 15 minutes before the beginning of the dark cycle, mice were given i.c.v. infusions of a 0.5-μg dose of MTII dissolved 0.9% saline (Experiment 4, $n = 8$; Experiment 5, $n = 12$) or an equal volume of 0.9% saline (Experiment 4, $n = 7$; Experiment 5, $n = 12$). The sweet solution, water, and food were returned immediately before the dark cycle. Intake measures were recorded 6 hours later.

Data Analyses

To obtain a measure that corrected for individual differences in body weight, grams of ethanol or food and milliliters of water or sweet solution consumed per kilogram of body weight were calculated. Ethanol preference ratios were also calculated by dividing the volume of ethanol consumed by total fluid (ethanol + water) consumption. Ethanol consumption data from Experiment 1 were analyzed with a 2 x 6 (genotype × ethanol concentration) repeated-measures analysis of variance (ANOVA), and saccharin and sucrose consumption data were analyzed with 2×2 (genotype × days) repeated-measures ANOVAs. Data from Experiments 2 and 3 were analyzed with two-way 2×3 (genotype × MTII dose) mixed-factor ANOVAs. Finally, data from Experiments 4 and 5 were analyzed using one-way (dose) ANOVAs. Tukey's tests were used for post hoc analyses. All data are presented as means ± SEM, and the level of significance was set at $p < 0.05$ in all cases.

RESULTS

Experiment 1: Two-Bottle Consumption of Ethanol and Water

Data showing 24-hour voluntary consumption of ethanol and water and ethanol preference ratios in $Mc4r^{-/-}$ and $Mc4r^{+/+}$ mice during 2-bottle testing are presented in Fig. 1. A repeated-measures ANOVA performed on ethanol consumption data revealed a significant main effect of ethanol concentration [$F(5,75) = 59.149$; $p = 0.001$], reflecting the increase in g/kg of ethanol consumed as the concentration of ethanol was increased over the course of the experiment (Fig. 1A). No other effects were statistically significant. A repeated-measures ANOVA performed on water consumption data revealed a significant main effect of ethanol concentration phase [$F(5,75) = 23.685$; $p = 0.001$], reflecting the greater consumption of water as the concentration of ethanol was increased. Interestingly, there was a significant main effect of genotype [$F(1,15) = 5.473$; $p = 0.034$], as $Mc4r^{+/+}$ mice (71.98 ± 5.24 ml/kg/24-h) drank significantly more water than $Mc4r^{-/-}$ mice (54.11 ± 5.56 ml/kg/24-h) over the course of the experiment (Fig. 1B). No other effects related to the water data were statistically significant. A repeated-measures ANOVA performed on ethanol preference ratio data revealed a significant main effect of ethanol concentration phase [$F(5,75) = 26.831$; $p = 0.001$], reflecting the reduced preference for ethanol solution relative to water as the concentration of ethanol was increased (Fig. 1C). Finally, a repeated-measures ANOVA comparing body weight data at each phase of the experiment revealed that there were no significant differences in body weight between $Mc4r^{+/+}$ mice (20.40 ± 1.09 g average over the course of the experiment) and $Mc4r^{-/-}$ mice (23.23 ± 1.11 g average over the course of the experiment).

A repeated-measures ANOVAs performed on saccharin consumption data revealed a significant effects of days [$F(1,22) = 8.627$; $p = 0.008$], reflecting increased consumption of

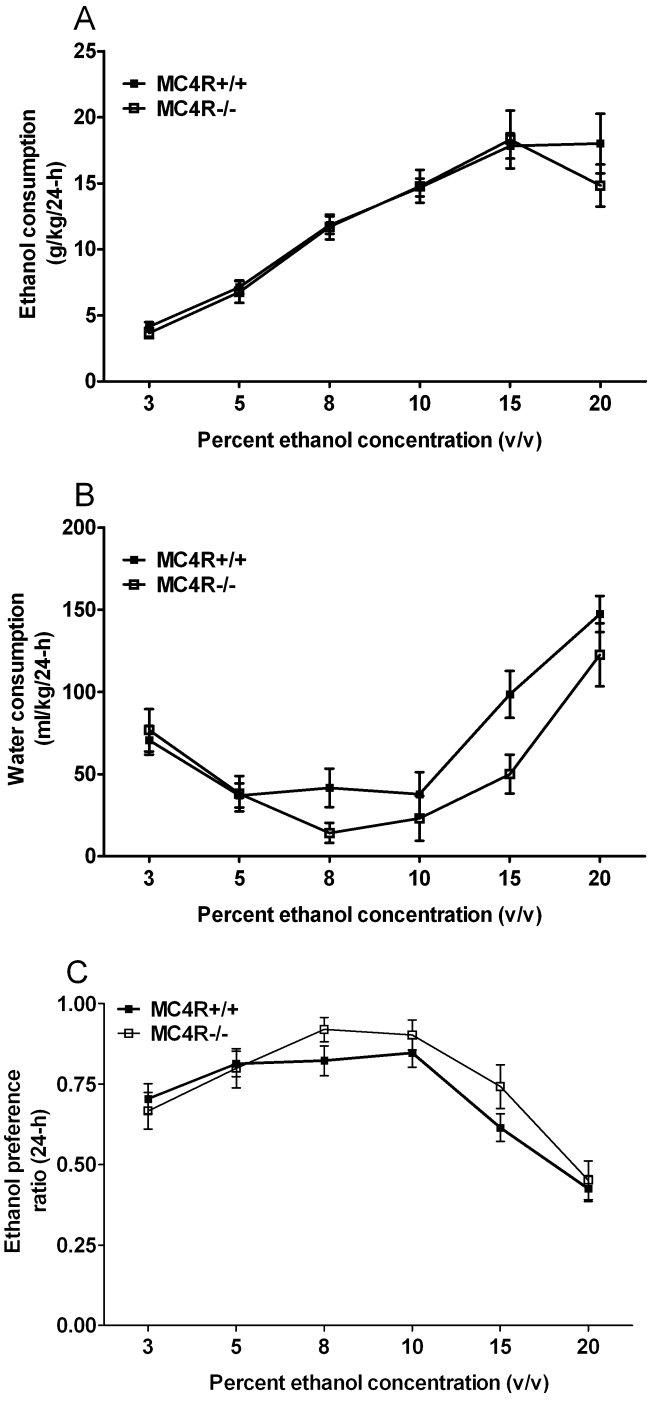

Fig. 1. Voluntary consumption of 3, 5, 8, 10, 15, and 20% (v/v) ethanol (panel **A**), water during access to different concentrations of ethanol (panel **B**), and ethanol preference ratios at each concentration of ethanol (panel **C**) in $Mc4r^{-/-}$ and $Mc4r^{+/+}$ mice in the two-bottle testing study (Experiment 1). All values are means ± SEM. $Mc4r^{+/+}$ mice drank significantly more water than $Mc4r^{-/-}$ mice over the course of the experiment as revealed by a significant main effect of genotype ($p = 0.001$).

0.15% saccharin over days. However, $Mc4r^{-/-}$ mice (289.66 ± 41.32 ml/kg/d) and $Mc4r^{+/+}$ mice (302.51 ± 35.24 ml/kg/d) did not differ significantly in the volume of saccharin solution consumed, nor were there any genotype differences in water intake during access to saccharin. Similarly, a repeated-measures ANOVAs performed on sucrose consumption data revealed a significant effect of days [$F(1,22) = 80.103$; $p = 0.001$], reflecting increased consumption of 10% sucrose over days. $Mc4r^{-/-}$ mice (390.50 ± 13.29 ml/kg/d) and $Mc4r^{+/+}$ mice (388.00 ± 26.44 ml/kg/d) did not differ significantly in the volume of sucrose solution consumed, nor were there any genotype differences in water intake during access to sucrose.

Experiment 2: Ethanol Consumption Following Central Infusion of the MCR Agonist MTII

Data showing 6-hour consumption measures following i.c.v. infusion of MTII in the $Mc4r^{-/-}$ and $Mc4r^{+/+}$ mice are presented in Fig. 2A–C. A two-way ANOVA performed on ethanol consumption data revealed a significant interaction effect between genotype and MTII dose [$F(2,39) = 3.739$; $p = 0.033$], but the genotype and MTII dose main effects were not significant. Post hoc tests showed that while each dose of MTII significantly reduced ethanol intake relative to control infusion in $Mc4r^{+/+}$ mice, neither dose tested altered ethanol intake in the $Mc4r^{-/-}$ mice (Fig. 2A). A two-way ANOVA performed on food intake data revealed a main effect of genotype [$F(1,39) = 6.854$; $p = 0.013$] and a significant interaction between genotype and MTII dose [$F(2,39) = 6.747$; $p = 0.003$] (Fig. 2B). Post hoc tests showed that while MTII was ineffective in $Mc4r^{-/-}$ mice, each dose of the agonist tested significantly reduced food intake (relative to vehicle treatment) in the $Mc4r^{+/+}$ mice. A two-way ANOVA performed on water intake data showed a significant interaction between genotype and MTII dose [$F(2,39) = 4.147$; $p = 0.023$], but the main effects were not statistically significant (Fig. 2C). Despite the significant interaction effect, post hoc tests revealed that MTII did not significantly alter water drinking relative to the vehicle treatment in either $Mc4r^{-/-}$ or $Mc4r^{+/+}$ mice. A two-way ANOVA performed on ethanol preference ratio data failed to show any significant effects (data not shown). Finally, a two-way ANOVA performed to compare body weight of mice in each treatment condition revealed that while there was a main effect of genotype [$F(1,39) = 10.020$; $p = 0.003$] such that $Mc4r^{+/+}$ mice (22.71 ± 0.93 g) weighed less than $Mc4r^{-/-}$ mice (26.667 ± 0.84 g), there was no significant interaction between genotype and MTII dose, suggesting that body weight did not likely contribute to the genotype × MTII dose interaction effects observed with ethanol consumption and food intake data. Increased body weight in $Mc4r^{-/-}$ mice has previously been reported (Huszar et al., 1997; Marsh et al., 1999).

Experiment 3: Ethanol Consumption Following Peripheral Administration of the MCR Agonist MTII

Data showing 6-hour consumption measures following i.p. injection of MTII in the $Mc4r^{-/-}$ and $Mc4r^{+/+}$ mice are presented in Fig. 2D–F. A two-way ANOVA performed on ethanol consumption data revealed a main effect of MTII

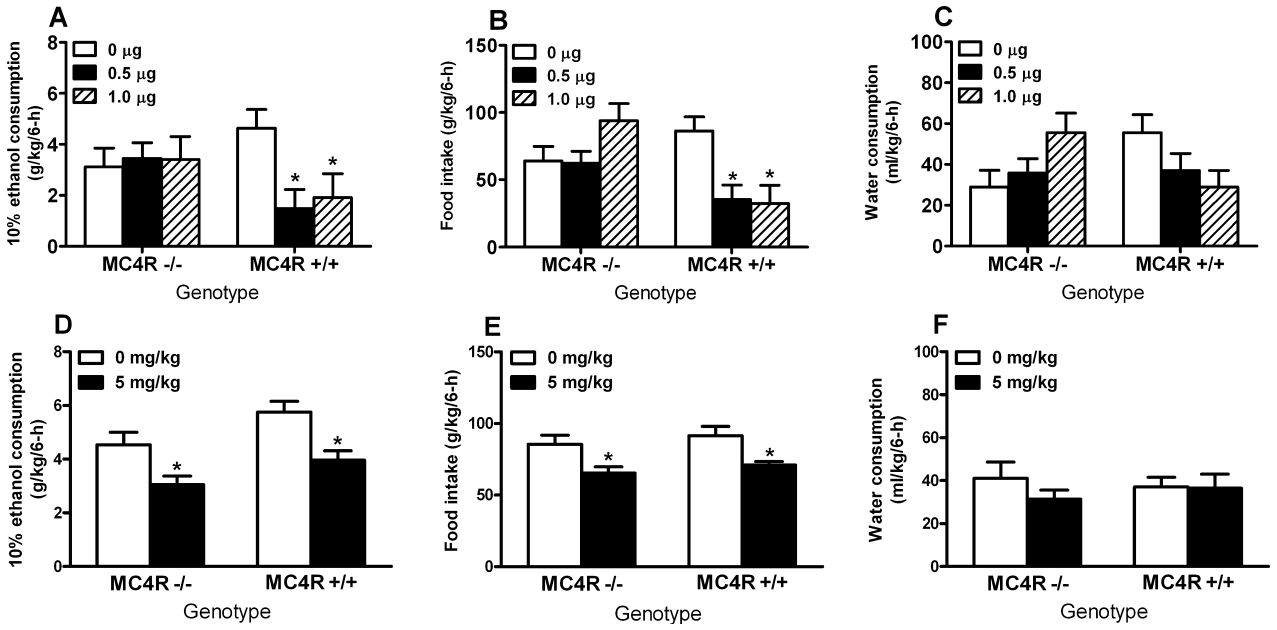

Fig. 2. Consumption of 10% (v/v) ethanol (g/kg/6-h), food (g/kg/6-h), and water (ml/kg/6-h) in $Mc4r^{-/-}$ and $Mc4r^{+/+}$ mice given intracerebroventricular infusion of saline (0 μg) or melanotan-II (MTII) (0.5 or 1.0 μg) are presented in panels **A**, **B**, and **C**, respectively (Experiment 2). Similarly, consumption of ethanol, food, and water in $Mc4r^{-/-}$ and $Mc4r^{+/+}$ mice given intraperitoneal injection of 0.9% saline (0 mg/kg) or MTII (5 mg/kg) are presented in panels **D**, **E**, and **F**, respectively (Experiment 3). All values are means ± SEM. *$p < 0.05$ relative to 0 μg or 0 mg/kg dose.

dose [$F(1,55) = 17.22$; $p = 0.001$]. Neither the genotype main effect nor the interaction effect was significant (Fig. 2D). A two-way ANOVA performed on food intake data revealed a main effect of MTII dose [$F(1,55) = 14.423$; $p = 0.001$], but the genotype main effect and interaction effect did not achieve statistical significance (Fig. 2E). A two-way ANOVA performed on water intake data failed to show any statistically significant effects (Fig. 2F). Similarly, a two-way ANOVA performed on ethanol preference ratio data failed to show any significant effects (data not shown). Finally, a two-way ANOVA performed to compare body weight of mice in each treatment condition revealed a main effect of genotype [$F(1,55) = 7.023$; $p = 0.011$] such that $Mc4r^{+/+}$ mice (23.38 ± 0.67 g) weighed less than the $Mc4r^{-/-}$ mice (25.89 ± 0.66 g). No other effects were significant.

Experiments 4 and 5: Sucrose and Saccharin Solution Consumption Following Central Infusion of the MCR Agonist MTII

Figure 3 shows data representing 6-h consumption measures during sucrose testing in Experiment 4 (Fig. 3A–C) and saccharin testing in Experiment 5 (Fig. 3D–F) in C57BL/6J mice that were given i.c.v. infusion of vehicle or a 0.5-μg dose of MTII. One-way ANOVAs performed on sucrose, food, and water intake data from Experiment 4 revealed that the 0.5-μg dose of MTII significantly reduced sucrose [$F(1,13) = 8.477$; $p = 0.012$] and food [$F(1,13) = 6.456$; $p = 0.025$] intake but did not significantly alter water drinking relative to the control condition. One-way ANOVAs performed on saccharin, food, and water intake data from Experiment 5 revealed that the 0.5-μg dose of MTII significantly reduced saccharin intake relative to the control injection [$F(1,22) = 7.622$; $p = 0.011$], but did not significantly alter food or water intake.

DISCUSSION

Constitutive deletion of the MC4R was not associated with significant alterations of voluntary ethanol consumption or consumption of saccharin or sucrose solutions (Experiment 1). An initial conclusion might be that endogenous MC4R signaling does not play a critical role in modulating ethanol self-administration. However, developmental compensation in constitutive knockout mice may mask the contribution of the deleted gene (Gerlai, 1996, 2001); thus, a role for endogenous MC4R signaling in modulating ethanol drinking cannot be ruled out by null data. Interestingly, consistent with a recent report implicating MCR signaling in the modulation of water intake (Yosten and Samson, 2010), the present data suggest that endogenous MC4R signaling may play a role in the modulation of water intake as $Mc4r^{+/+}$ mice drank more water than $Mc4r^{-/-}$ mice over the course of Experiment 1. Importantly, i.c.v. infusion of MTII (0.5- and 1.0-μg doses) significantly reduced 6-hour ethanol consumption and food intake in $Mc4r^{+/+}$ mice without significantly altering water drinking, but failed to influence ethanol drinking or feeding in $Mc4r^{-/-}$ mice (Experiment 2). These observations support previous findings showing that MTII significantly reduces ethanol intake in C57BL/6J mice (Navarro et al., 2003, 2005) and extend the literature by showing that the MC4R is the primary receptor involved in

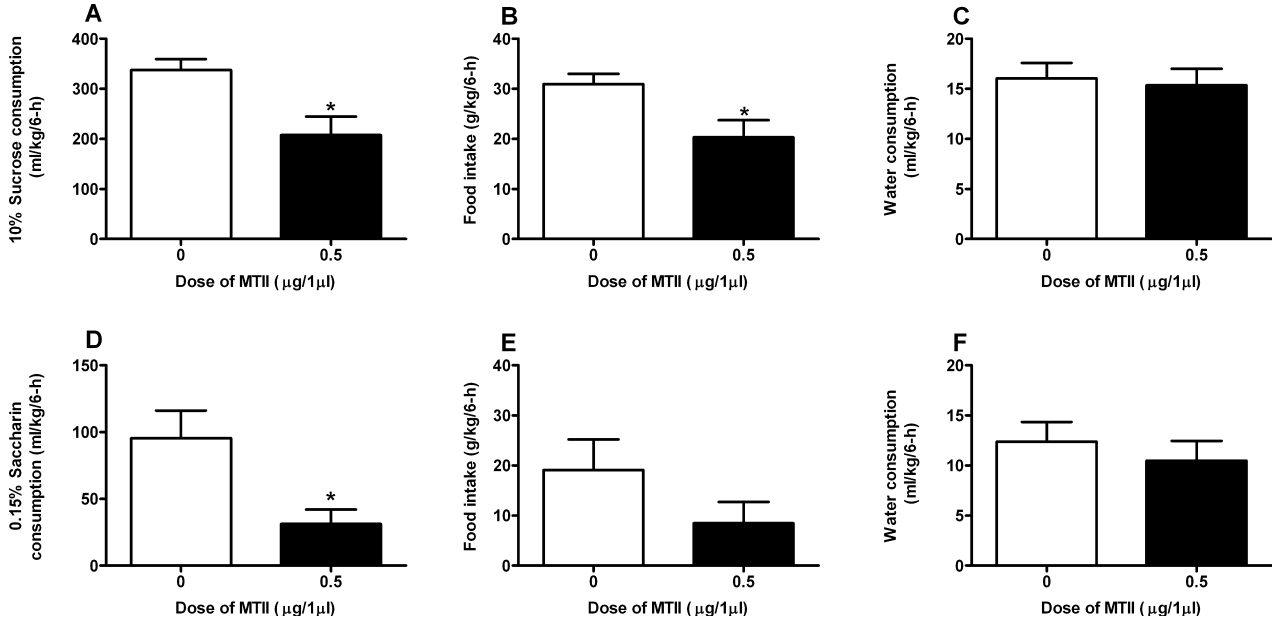

Fig. 3. Consumption of sweet solution (ml/kg/6-h; panels **A** and **D**), food (g/kg/6-h; panels **B** and **E**), and water (ml/kg/6-h; panels **C** and **F**) in C57BL/6J mice given intracerebroventricular infusion of saline (0 μg) or melanotan-II (0.5 μg). Sweet solution was made from 10% sucrose (Experiment 4; panels **A**, **B**, and **C**) or 0.15% saccharin (Experiment 4; panels **D**, **E**, and **F**). All values are means ± SEM. *$p < 0.05$ relative to 0-μg dose.

modulating the protective effects of centrally infused MTII on excessive ethanol intake. The present findings also replicate previous work demonstrating that central administration of MTII attenuates food intake (Grill et al., 1998; Hollopeter et al., 1998; Marsh et al., 1999; Navarro et al., 2003, 2005; Pierroz et al., 2002) and requires the MC4R (Marsh et al., 1999). Together, the present work highlights the critical role of the MC4R in modulating the central pharmacological effects of the MCR agonist MTII on ethanol intake and feeding. On the other hand, as Mc3r$^{-/-}$ mice showed normal ethanol drinking and food intake when MTII was centrally infused (Navarro et al., 2005), the MC3R does not appear to be involved.

Consistent with previous reports (Cettour-Rose and Rohner-Jeanrenaud, 2002; Navarro et al., 2003, 2005; Pierroz et al., 2002), here we show that the peripherally administered MTII (5 mg/kg) reduced ethanol drinking and food intake. However, unlike central administration, when administered peripherally, MTII did not require normal MC4R expression to suppress feeding or ethanol intake. This conclusion is supported by the observations that i.p. injection of MTII significantly reduced 6-hour ethanol consumption and food intake (but not water drinking) with similar effectiveness in Mc4r$^{-/-}$ and Mc4r$^{+/+}$ mice (Experiment 3). Because the MC4R is necessary for the central actions of MTII, the present data suggest that the effects of peripherally administered MTII on ethanol drinking and food intake may be modulated by other MCRs. A possibility is that peripheral MCRs (other than the MC4R) are involved. In fact, radiolabeled MTII, when given in an intravenous injection at a dose that attenuated food intake, was evident in the circumventricular organs but did not readily penetrate the blood–brain barrier in rats (Trivedi et al., 2003), and a more recent study showed low penetration of peripherally administered MTII into mouse brain (Hatziieremia et al., 2007). MC immunoreactivity and MC receptor binding have been observed in peripheral tissues, including the gastrointestinal tract and the adrenal glands (Dhillo et al., 2005; Saito et al., 1983; Tatro and Reichlin, 1987), and it is therefore possible that peripherally administered MTII attenuated ethanol consumption and food intake by actions within these peripheral regions. It should be noted that while a previous report showed that an i.p. injection of a 10 mg/kg dose of MTII reduced food intake in both Mc4r$^{-/-}$ and Mc4r$^{+/+}$ mice (Chen et al., 2000), a more recent finding showed that an i.p. injection of a 100-μg dose of MTII failed to alter feeding in Mc4r$^{-/-}$ mice but was effective in Mc4r$^{+/+}$ mice (Balthasar et al., 2005). Thus, it is also possible that lower doses of peripherally administered MTII require the MC4R to reduce food (and ethanol) intake, while higher doses (such as the 5 mg/kg dose used here) influence ingestive behaviors by acting on other MCRs. A more comprehensive assessment of the effects of peripherally administered MCR agonists, over a range of doses, on ethanol intake (as well as possible nonspecific effects) will be the focus of future research.

One goal of the present report was to assess the effects of MTII on the consumption of various reinforcing stimuli, in addition ethanol and food. I.c.v. infusion of a 0.5-μg dose of MTII, which significantly reduced 6-hour ethanol drinking and food intake in wild-type mice, also attenuated 6-hour consumption of a 10% sucrose solution and a 0.15% saccharin solution without altering water drinking. Thus, the MCR agonist MTII blunts the consumption of both caloric (ethanol, food, and sucrose) and noncaloric (saccharin) reinforcers, observations that are consistent with the hypothesis

that overlapping MC pathways modulate ethanol consumption and the consumption of natural reinforcers, regardless of caloric content. In fact, this should not come as a surprise in light of electrophysiological evidence demonstrating that both drugs of abuse and "natural" reinforcers (food and water) produce similar cell firing in the NAc (Carelli et al., 2000; Hollander et al., 2002; Roitman et al., 2004, 2005, 2008; Roop et al., 2002), and the observation that a growing list of peptides and proteins modulate both ethanol consumption and food intake (Thiele et al., 2003). For example, opioid receptor antagonists, which are approved for treating alcoholism, reduce both ethanol consumption and food intake (Gonzales and Weiss, 1998; Kamdar et al., 2007; Kotz et al., 1997; Middaugh et al., 2000; Yeomans and Gray, 2002). Interestingly, it has been proposed that cannabinoid receptor (CB1) agonists may be useful therapeutic agents for treating obesity (Cota et al., 2003) and alcoholism (Racz et al., 2003), and we suggest that MCR agonists may also provide a dual therapeutic role.

Given that administration of MTII was associated with reduced consumption of each of the reinforcing stimuli examined here, one potential concern is that administration of MTII produces nonspecific, and potentially aversive, effects. However, contrary to this hypothesis is the observation that MTII failed to significantly alter water intake relative to vehicle treatment in each of the experiments reported here, and we have previously observed MTII-induced attenuation of ethanol drinking that was not associated with altered water intake (Navarro et al., 2003, 2005). Another potential concern is that the effects of MCR agonists on ethanol drinking may be secondary to alterations of ethanol metabolism. However, this is unlikely because we have previously shown that peripheral administration and central administration of MTII do not alter blood ethanol clearance (Navarro et al., 2003, 2005).

Interestingly, while not significant when compared to the vehicle condition, there was a trend for the 1.0-μg dose of MTII to increase food intake in $Mc4r^{-/-}$ mice in Experiment 2. MCR agonist-induced increase in food intake has previously been reported in $Mc4r^{-/-}$ mice and was hypothesized to reflect a compensatory increase in MC3R signaling (Kumar et al., 2009). Consistent with this idea, a selective MC3R agonist was found to increase food intake, suggesting that the MC3R functions as a presynaptic autoreceptor in brain regions that modulate food intake (Cone, 2006; Marks et al., 2006).

In conclusion, the present work provides new insight into the mechanism by which MCR signaling influences ethanol consumption and feeding by demonstrating the essential role of central MC4R in modulating MCR agonist-induced reductions of ethanol intake and food intake. On the other hand, the MC4R does not modulate the effects of peripherally administered MCR agonist (MTII) on ethanol and food intake, suggesting that different populations of MCRs modulate the actions of centrally versus peripherally administered MTII. Centrally administered MTII also attenuated the consumption of sucrose and saccharin solutions at a dose that did not alter water drinking, consistent with the hypothesis that overlapping central MC pathways modulate the reinforcing properties of ethanol and natural reinforcers, independent of caloric content. Taken together, the present observations and previous work suggest that MC4R agonists, in addition to being attractive targets for treating obesity, may have therapeutic value for treating excessive ethanol consumption in individuals afflicted with alcohol abuse disorders or that are ethanol dependent.

ACKNOWLEDGMENTS

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Ethanol-Induced Increase of Agouti-Related Protein (AgRP) Immunoreactivity in the Arcuate Nucleus of the Hypothalamus of C57BL/6J, but not 129/SvJ, Inbred Mice

Inmaculada Cubero, Montserrat Navarro, Francisca Carvajal, Jose Manuel Lerma-Cabrera, and Todd E. Thiele

Background: The melanocortin (MC) system is composed of peptides that are cleaved from the polypeptide precursor, pro-opiomelanocortin (POMC). Previous research has shown that MC receptor (MCR) agonists reduce, and MCR antagonists increase, ethanol consumption in rats and mice. Consistently, genetic deletion of the endogenous MCR antagonist, agouti-related protein (AgRP), causes reductions of ethanol-reinforced lever pressing and binge-like ethanol drinking in C57BL/6J mice. Ethanol also has direct effects on the central MC system, as chronic exposure to an ethanol-containing diet causes significant reductions of α-melanocyte stimulating hormone (α-MSH) immunoreactivity in specific brain regions of Sprague-Dawley rats. Together, these observations suggest that the central MC system modulates neurobiological responses to ethanol. To further characterize the role of the MC system in responses to ethanol, here we compared AgRP and α-MSH immunoreactivity in response to an acute injection of saline or ethanol between high ethanol drinking C57BL/6J mice and moderate ethanol drinking 129/SvJ mice.

Methods: Mice received an intraperitoneal (i.p.) injection of ethanol (1.5 g/kg or 3.5 g/kg; mixed in 0.9% saline) or an equivolume of 0.9% saline. Two hours after injection, animals were sacrificed and their brains were processed for AgRP and α-MSH immunoreactivity.

Results: Results indicated that acute ethanol administration triggered a dose-dependent increase in AgRP immunoreactivity in the arcuate (ARC) of C57BL/6J mice, an effect that was not evident in the 129/SvJ strain. Although acute administration of ethanol did not influence α-MSH immunoreactivity, C57BL/6J mice had significantly greater overall α-MSH immunoreactivity in the ARC, dorsomedial, and lateral regions of the hypothalamus relative to the 129/SvJ strain. In contrast, C57BL/6J mice displayed significantly lower α-MSH immunoreactivity in the medial amygdala.

Conclusions: The results show that acute ethanol exposure has direct effects on endogenous AgRP activity in ethanol preferring C57BL/6J mice. It is suggested that ethanol-induced increases in AgRP may be part of a positive feedback system that stimulates excessive binge-like ethanol drinking in C57BL/6J mice. Inherent differences in α-MSH immunoreactivity may contribute to differences in neurobiological responses to ethanol that are characteristically observed between the C57BL/6J and 129/SvJ inbred strains of mice.

Key Words: α-MSH, AgRP, Immunoreactivity, 129/SvJ, C57BL/6J, Ethanol, Arcuate Nucleus, Medial Amygdala.

THE MELANOCORTIN (MC) system is composed of peptides that are cleaved from the polypeptide precursor pro-opiomelanocortin (POMC). Central MC peptides are produced by neurons within the hypothalamic arcuate (ARC) nucleus and the medulla (Dores et al., 1986; Jacobowitz and O'Donohue, 1978; O'Donohue and Dorsa, 1982), and include adrenocorticotropic hormone, α-melanocyte stimulating hormone (α-MSH), β-MSH, and γ-MSH (Hadley and Haskell-Luevano, 1999). Due to a lack of a critical dibasic site, β-MSH is not processed in rodent brain (Pritchard et al., 2002). In rodents, MC peptides act through 5 MC receptors (MC1R to MC5R) (Hadley and Haskell-Luevano, 1999). The MC3R and MC4R are the primary MC receptors (MCR) expressed in the rodent brain (Alvaro et al., 1997). Agouti-related protein (AgRP), a neuropeptide produced in the hypothalamus and co-secreted with neuropeptide Y in the same synaptic complexes as α-MSH functions as a natural MCR antagonist (Shutter et al., 1997). More recent in vivo evidence suggests that AgRP may function as a MCR inverse agonist (Tolle and Low, 2008).

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β-endorphin, an opioid peptide that is also cleaved from POMC and is co-expressed with α-MSH in the brain (Bloch et al., 1979), modulates neurobiological responses to ethanol (Froehlich and Li, 1993; Gianoulakis, 2001; Rasmussen et al., 2002; Scanlon et al., 1992; Zhou et al., 2000) and opioid receptor antagonists reduce ethanol consumption (Gianoulakis, 2001). Interestingly, there appear to be a functional interaction between MC and opioid neuropeptides. For example, stimulation of MCRs block the antinociceptive effects of opioids, whereas MC4R antagonists enhance opioid antinociception (Ercil et al., 2005). It is therefore not surprising that evidence has emerged suggesting that MC neuropeptides also modulate neurobiological responses to ethanol. First, α-MSH is expressed in brain regions implicated in ethanol's effects, including the striatum, nucleus accumbens (NAc), ventral tegmental area (VTA), amygdala, hippocampus, and hypothalamus (Bloch et al., 1979; Dube et al., 1978; Jacobowitz and O'Donohue, 1978; O'Donohue and Jacobowitz, 1980; O'Donohue et al., 1979; Yamazoe et al., 1984). Second, rats selectively bred for high ethanol drinking (AA [Alko, Alcohol]) have low levels of MC3R in the shell of the NAc, but have high levels of MC3R and MC4R in various regions of the hypothalamus, when compared with low ethanol-drinking rats (Lindblom et al., 2002). Third, central infusion of the nonselective MCR agonist, melanotan-II (MTII) significantly reduced voluntary ethanol drinking in AA rats with an established history of ethanol intake (Ploj et al., 2002). Similarly, MTII-induced reduction of ethanol consumption was shown to be receptor-mediated and not associated with alterations of ethanol metabolism in C57BL/6J mice (Navarro et al., 2003). More recently, ventricular infusion of a selective MC4R agonist significantly reduced ethanol drinking, whereas ventricular infusion of the nonselective MCR antagonist AgRP-(83 to 132) significantly increased ethanol drinking, by C57BL/6J mice (Navarro et al., 2005). Consistent with pharmacological data, genetic deletion of endogenous AgRP reduced ethanol-reinforced lever pressing and binge-like ethanol drinking in C57BL/6J (Navarro et al., 2009). Ethanol also has direct effects on central MC activity. Thus, chronic exposure to ethanol significantly reduced (Navarro et al., 2008), while abstinence following chronic ethanol exposure increased (Kokare et al., 2008), endogenous α-MSH immunoreactivity in specific brain regions of Sprague-Dawley rats.

The objective of this study was to further characterize the roles of endogenous AgRP and α-MSH in the modulation of neurobiological responses to ethanol. Many genetically altered mouse lines, including MC3R-, MC4R-, and AgRP-deficient mice (Marsh et al., 1999; Navarro et al., 2005, 2009), were originally derived on a hybrid genetic background of the C57BL/6J inbred strain and 1 of the 129 substrains. Thus, assessing differences in AgRP and α-MSH immunoreactivity between the C57BL/6J and a 129 strain would provide valuable informative for studies that examine ethanol-related phenotypes in MCR or AgRP knockout mice, or in other knockout mice which entail mutations that interact with the MC system. Furthermore, comparisons of inbred strains of mice is a useful first-step approach to identify neurochemical pathways that may modulate neurobiological responses to ethanol and ethanol intake (Bachtell et al., 2002; Hayes et al., 2005; Weitemier et al., 2005). Thus, here we compared brain AgRP and α-MSH immunoreactivity between C57BL/6J and 129/SvJ mice inbred strains that have been shown to exhibit different levels of ethanol consumption. C57BL/6J mice voluntarily consume 10 g/kg/d to 12 g/kg/d of ethanol when offered a 10% (v/v) solution, while mice of the 129/SvJ strain consumes about half as much, or 5 g/kg/d to 6 g/kg/d of 10% ethanol (Belknap et al., 1993). Based on previous pharmacological and genetic observations, we predicted that these inbred strains would show differences in baseline and ethanol-induced AgRP and α-MSH immunoreactivity in brain region implicated in neurobiological responses to ethanol.

MATERIALS AND METHODS

Animals

Male 129/SvJ and C57BL/6J inbred strains of mice (Jackson Laboratories, Bar Harbor, ME) weighed approximately 20 g to 25 g and were 6 to 8 weeks of age upon arrival. Mice were individually housed in polypropylene cages with wood-chip bedding and had ad libitum access to standard rodent chow (Teklad, Madison, WI) and water throughout the experiment. The colony room was maintained at approximately 22°C with a 12 hours:12 hours, light:dark cycle. All procedures used in this study were in compliance with the National Institute of Health guidelines, and all protocols were approved by the University of North Carolina Institutional Animal Care and Use Committee. Body weight (g), water (ml/kg/24 h), and food (g/kg/24 h) consumption were collected for 1 week prior to the experiment and average baseline measures were calculated for each strain of inbred mice.

Acute Ethanol Administration

On the test day, mice were weighed, and water and food were removed from each cage. Then, mice were divided into 3 groups based on body weight, and given a single intraperitoneal (i.p.) injection of ethanol (1.5 g/kg or 3.5 g/kg, 7.5 ml/kg and 17.5 ml/kg, respectively; 20% [w/v] mixed in 0.9% saline) or an equivolume of 0.9% saline (17.5 ml/kg). Mice were then returned to their homecage immediately after the injection where they remained until perfusion procedures. As α-MSH and AgRP have been implicated in feeding behaviors (Sainsbury et al., 2002), procedures were carried out during the animals' light cycle, a time of day in which feeding behavior in mice is low. Injections began 2 hours into the light cycle, and were staggered (counterbalanced by strain and injection condition) so that all mice were perfused exactly 2 hours after injection. Another set of naive C57BL/6J and 129/SvJ mice were given i.p. injection of a 1.5 g/kg or 3.5 g/kg dose of ethanol, and blood samples were collected 2 hours later to assure that ethanol-induced differences in immunohistochemistry (IHC) between strains were not associated with strain differences in blood ethanol concentrations (BECs). Approximately 10 μl of blood was collected from the tail vein of each mouse, samples were centrifuged, and 5 μl of plasma from each sample was analyzed for BECs measured in mg/dl (Analox Instruments, Lunenburg, MA).

Perfusions, Brain Preparation, and Immunohistochemistry

Two hours after ethanol or saline injection, mice were injected with a cocktail of ketamine (117 mg/kg) and xylazine (7.92 mg/kg) and then transcardially perfused for 10 minutes with 0.1 mM

phosphate-buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde in phosphate buffer. The 2 hours postinjection perfusion time was chosen because we have previously found treatment-induced differences in protein at this time interval (Thiele et al., 1997, 1998a, 2000). All perfusions were completed within a 5-hour window of time. The brains were collected and postfixed in paraformaldehyde for 24 hours at 4°C, at which point they were transferred to PBS. Brains were cut using a vibrotome into 40 μm sections and were then stored in PBS. IHC procedures were based on those routinely used in our laboratory (Hayes et al., 2005; Thiele et al., 1996, 1997, 1998a,b, 2000). Sections were evenly divided into 2 sets (every-other section) for processing with α-MSH or AgRP antibodies. After rinsing in fresh PBS 4 times (10 minutes each), tissue sections were blocked in 10% rabbit serum (for α-MSH) or 10% goat serum (for AgRP) and 0.1% triton-X-100 in PBS for 1 hour. Sections were then transferred to fresh PBS containing primary sheep anti-α-MSH (Millipore, Billerica, MA; 1:10,000) or primary rabbit anti-AgRP (Phoenix Pharmaceuticals, Inc., Burlingame, CA; 1:4,000) for 3 days at 4°C. As a control to determine if staining required the presence of the primary antibodies, some sections were run through the assay without primary antibody (α-MSH or AgRP). In each assay described below, tissue processed without the primary antibody failed to show staining that was evident in tissue processed with primary antibody. After the 3 days of incubation, the sections were rinsed 4 times and then processed with Vectastain Elite kits (Vector Labs, Burlingame, CA) as per the manufacturer's instructions for standard ABC/HRP/diaminobenzidinebased IHC. The sections processed for α-MSH or AgRP were visualized by reacting the sections with a 3,3-diaminobenzidine tetrahydrochloride (DAB; Polysciences, Inc., Warrington, MA) reaction solution containing 0.05% DAB, 0.005% cobalt, 0.007% nickel ammonium sulfate, and 0.006% hydrogen peroxide. All sections were mounted on glass slides, air-dried overnight, and cover slipped for viewing. Digital images of α-MSH and AgRP IHC were obtained on a Nikon E400 microscope equipped with a Nikon Digital Sight DS-U1 digital camera run with Nikon-provided software. For analysis, great care was taken to match sections through the same region of brain and at the same level using anatomic landmarks with the aid of a mouse stereotaxic atlas (Franklin and Paxinos, 1997). Densitometric procedures were used to assess protein levels. Flat-field corrected digital pictures (8-bit grayscale) were taken using the Digital Sight DS-U1 camera and density of staining was analyzed using Image J software (Image J; National Institute of Health, Bethesda, MD) by calculating the percent of the total area examined that showed signal (cell bodies and processes) relative to a subthreshold background. The size of the areas that were analyzed was the same between animals and groups. The subthreshold level for the images was set in such a way that any area without an experimenter-defined level of staining (determined by terminal- and/or soma-positive regions) was given a value of zero. Within each region, the same subthreshold level was used for each slice that was scored. Data from each brain region in an animal were calculated by taking the average counts from 2 brain slices. Data from each slice were calculated by taking the average counts from the left and right sides of the brain at the specific brain region of interest. For each brain region, photographs were taken at approximately the medial area of the structure (with respect to the rostral-caudal axis) for each of the 2 brain slices that were scored. In all cases, quantification of IHC data was conducted by an experimenter that was blinded to group identity. We quantified α-MSH immunoreactivity in regions of the hypothalamus (the ARC, dorsomedial [DMH], and lateral [LH] regions) and the medial amygdala (MA) regions in which we found robust α-MSH staining and which have been implicated in neurobiological responses to ethanol and other drugs of abuse (Aston-Jones et al., 2009; Cannella et al., 2009; DiLeone et al., 2003; Doron et al., 2006; Harris and Aston-Jones, 2006; Hunt and McGregor, 1998; Koob, 2003). Consistent with our previous findings (Navarro et al., 2008), AgRP immunoreactivity was observed primarily in the ARC.

Data Analyses

All data collected in this study are presented as mean ± SEM and differences between groups were analyzed using analysis of variance (ANOVA). One-way ANOVAs were performed on baseline body weight, food intake, and water consumption data. Independent two-way, 2 × 3 (strain × dose) ANOVAs were performed on IHC data collected from each brain region and on BEC data. Sample sizes vary in IHC analyses below as appropriate representative sections through target brain regions were not available in some cases. When significant main effects or interactions were found, post hoc analyses were conducted using Newman–Keuls tests. To control for the increased likelihood of Type 1 errors with multiple comparisons, the level of significance was set at $p < 0.0125$ for analyses involving α-MSH immunoreactivity data in the 4 brain regions examined, consistent with a Bonferroni correction. For all other analyses, the level of significance was set at $p < 0.05$.

RESULTS

Strain Comparisons of Body Weight, Food and Water Intake, and Blood Ethanol Levels

Because α-MSH and AgRP have been implicated in food intake and body weight regulation (Sainsbury et al., 2002), we determined if there were differences between C57BL/6J and 129/SvJ mice in body weight, food intake, and water drinking. The C57BL/6J mice ($n = 25$) and 129/SvJ mice ($n = 25$) did not differ significantly in average body weight ([$F(1, 48) = 0.2$, $p > 0.05$]; 20.6 ± 0.14 g and 20.4 ± 0.14 g, respectively), food intake ([$F(1, 48) = 0.4$, $p > 0.05$]; 4.5 ± 0.14 g/d and 4.6 ± 0.14 g/d, respectively), or water drinking [$F(1, 48) = 0.2$, $p > 0.05$]; 5.2 ± 0.14 ml/d and 5.1 ± 0.14 ml/d, respectively). Additionally, C57BL/6J ($n = 7$ per dose condition) and 129/SvJ ($n = 6$ or 7 for the 1.5 g/kg and 3.5 g/kg doses of ethanol, respectively) mice did not differ significantly in BECs 2 hours following injection of a 1.5 g/kg (51.11 ± 4.48 mg/dl and 71.03 ± 4.49 mg/dl, respectively) or a 3.5 g/kg (273.12 ± 3.51 mg/dl and 277.96 ± 16.36 mg/dl, respectively) dose of ethanol. A two-way ANOVA performed on BEC data showed a significant main effect of ethanol dose [$F(1, 23) = 538.02$, $p < 0.05$] but neither the strain main effect [$F(1, 23) = 1.79$, $p > 0.05$] nor interaction effect [$F(1, 23) = 0.66$, $p > 0.05$] were statistically significant.

AgRP IHC in the Arcuate Nucleus Following Saline or Ethanol Injection

Figure 1 shows data representing the average densities of AgRP immunoreactivity in the ARC of 129/SvJ and C57BL/6J mice given i.p. injection of saline ($n = 6$ and 4, respectively), a 1.5 g/kg dose of ethanol ($n = 7$ and 4, respectively) or a 3.5 g/kg dose of ethanol ($n = 5$ and 7, respectively), and representative photomicrographs of AgRP immunoreactivity in the ARC of 129/SvJ and C57BL/6J mice are presented in Fig. 2. A two-way ANOVA performed on this data set showed a statistically significant main effect of mouse strain [$F(1, 27) = 14.15$, $p < 0.05$] and a significant

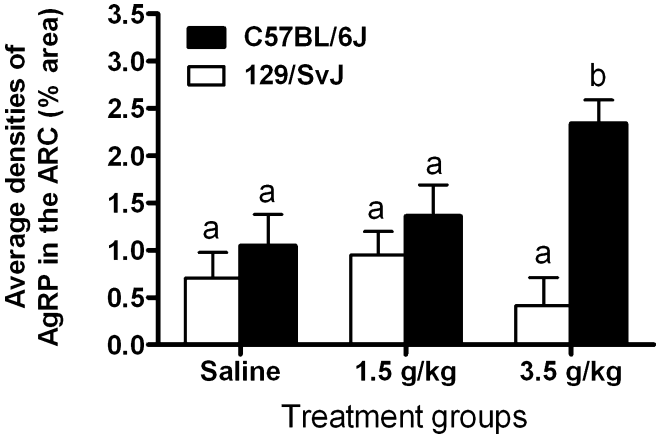

Fig. 1. Quantification of Agouti-related protein immunoreactivity (AgRP) (% area) in the arcuate nucleus of the hypothalamus (ARC). 129/SvJ and C57BL/6J mice were given intraperitoneal injection of isotonic saline or a 1.5 g/kg or 3.5 g/kg dose of ethanol, and brains were collected 2 hours after injections. Values are represented as mean ± SEM. There are statistical differences between groups that do not share overlapping lettering (a or b; $p < 0.05$).

dose × mouse strain interaction [$F(2, 27) = 5.0$, $p < 0.05$], but the dose main effect was not statistically significant [$F(1, 27) = 1.5$, $p > 0.05$]. Post hoc Newman–Keuls tests showed that C57BL/6J mice exhibited a dose-dependent increase in AgRP immunoreactivity that was not evident in 129/SvJ mice.

α-MSH IHC in Regions of the Hypothalamus and MA Following Saline or Ethanol Injection

Arcuate Nucleus. Figure 3*A* shows data representing the average densities of α-MSH immunoreactivity in the ARC of 129/SvJ and C57BL/6J mice given i.p. injection of saline ($n = 4$/strain), a 1.5 g/kg dose of ethanol ($n = 8$ and 5, respectively) or a 3.5 g/kg dose of ethanol ($n = 6$ and 7, respectively), and representative photomicrographs of α-MSH immunoreactivity in the ARC of 129/SvJ and C57BL/6J mice are presented in Fig. 4*A*,*E*, respectively. A two-way ANOVA performed on these data revealed a significant main effect of mouse strain [$F(1, 28) = 7.4$, $p < 0.0125$]. Neither the dose effect [$F(2,28) = 0.6$, $p > 0.0125$] nor the dose × strain interaction [$F(2, 28) = 0.4$, $p > 0.0125$] attained statistical significance.

Lateral Hypothalamus. Figure 3*B* shows data representing the average densities of α-MSH immunoreactivity in the LH of 129/SvJ and C57BL/6J mice given i.p. injection of saline ($n = 8$/strain), a 1.5 g/kg dose of ethanol ($n = 8$/strain) or a 3.5 g/kg dose of ethanol ($n = 8$ and 9, respectively), and representative photomicrographs of α-MSH immunoreactivity in the LH of 129/SvJ and C57BL/6J mice are presented in Fig. 4*B*,*F*, respectively. A two-way ANOVA performed on this data set showed a significant main effect of mouse strains [$F(1, 43) = 39.2$, $p < 0.0125$]. The dose main effect [$F(2, 43) = 0.4$, $p > 0.0125$] and the interaction effect [$F(2, 43) = 0.7$, $p > 0.0125$] were not statistically significant.

Dorsomedial Hypothalamus. Figure 3*C* shows data representing the average densities of α-MSH immunoreactivity in the DMH of 129/SvJ and C57BL/6J mice given i.p. injection of saline ($n = 7$/strain), a 1.5 g/kg dose of ethanol ($n = 6$ and 8, respectively) or a 3.5 g/kg dose of ethanol ($n = 7$ and 8, respectively), and representative photomicrographs of α-MSH immunoreactivity in the DMH of 129/SvJ and C57BL/6J mice are presented in Fig. 4*C*,*G*, respectively. A

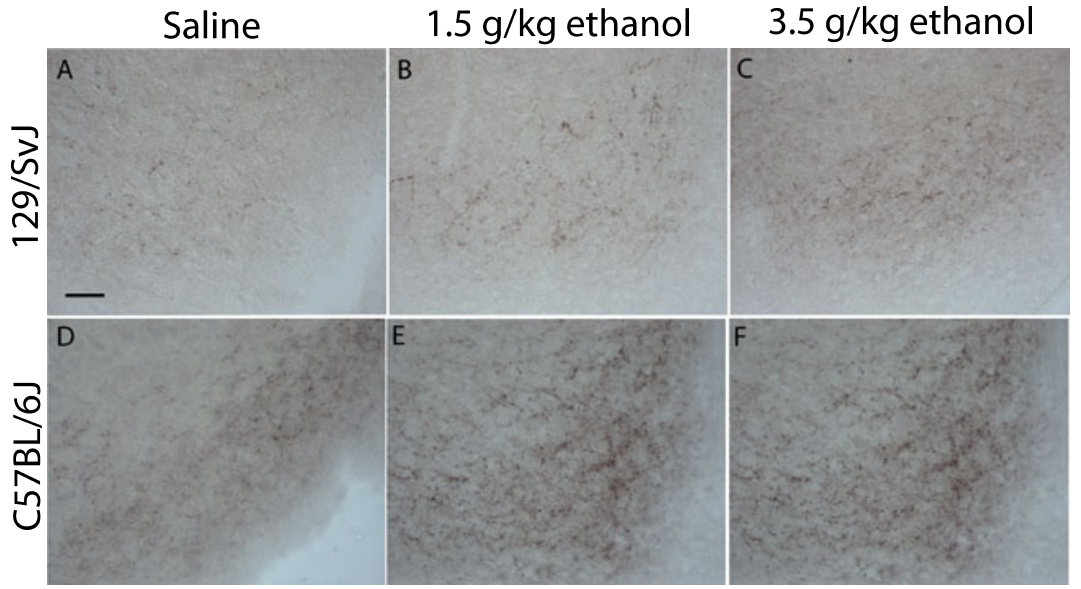

Fig. 2. Representative photomicrographs of 40 μm coronal sections showing Agouti-related protein immunoreactivity through the arcuate nucleus of the hypothalamus (ARC) in 129/SvJ and C57BL/6J mice that were given intraperitoneal injection of isotonic saline or a 1.5 g/kg or 3.5 g/kg dose of ethanol. Images were photographed at approximately −1.7 mm relative to Bregma and quantified at a magnification of 40×. Scale bar = 50 μm.

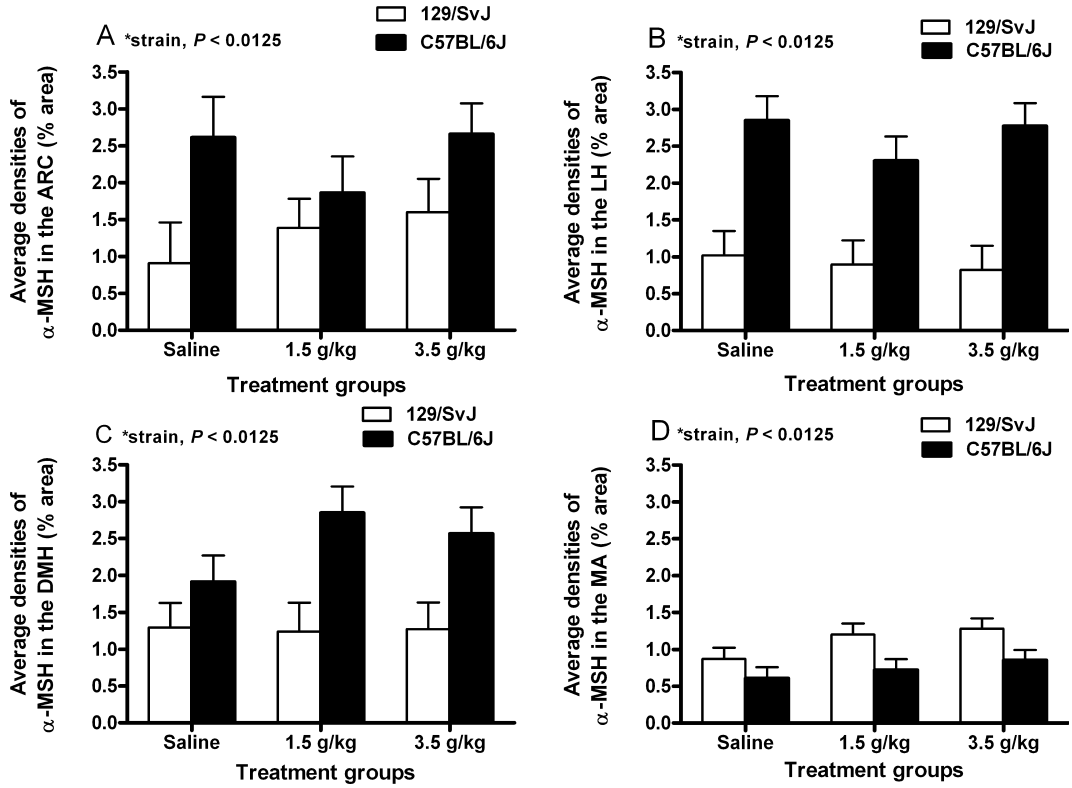

Fig. 3. Quantification of α-melanocyte stimulating hormone (α-MSH) immunoreactivity (% area) in the arcuate nucleus of the hypothalamus (ARC; **A**), the lateral nucleus of the hypothalamus (LH; **B**), the dorsomedial nucleus of the hypothalamus (DMH; **C**), and the medial amygdala (MA; **D**). 129/SvJ and C57BL/6J mice were given intraperitoneal injection of isotonic saline or a 1.5 g/kg or 3.5 g/kg dose of ethanol, and brains were collected 2 hours after injections. Values are represented as mean ± SEM.

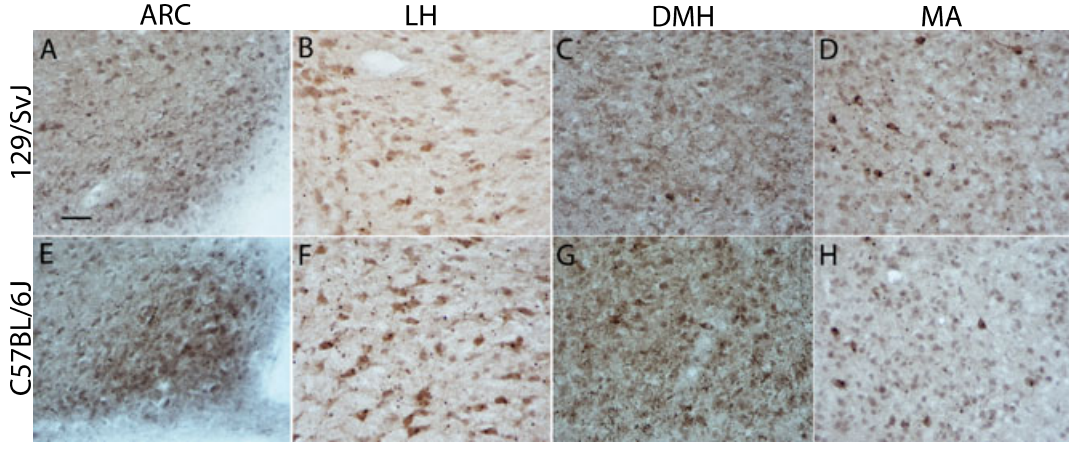

Fig. 4. Representative photomicrographs of 40 μm coronal sections showing α-melanocyte stimulating hormone (α-MSH) immunoreactivity through the arcuate nucleus of the hypothalamus (ARC; **A** and **E**), the lateral nucleus of the hypothalamus (LH; **B** and **F**), the dorsomedial nucleus of the hypothalamus (DMH; **C** and **G**), and the medial amygdala (MA; **D** and **H**) in saline treated mice. Sections were collected from 129/SvJ (**A–D**) and C57BL/6J (**E–H**) mice. Images were photographed and quantified at a magnification of 40×. Relative to Bregma, photographs were taken at approximately −1.7 mm (ARC and DMH) and −1.34 mm (LH and MA). Scale bar = 50 μm.

two-way ANOVA performed on these data showed a statistically significant main effect of mouse strains [$F(1, 37) = 10.7$, $p < 0.0125$], but neither the dose [$F(2, 37) = 0.7$, $p > 0.0125$] nor the interaction [$F(2, 37) = 0.8$, $p > 0.0125$] effects were statistically significant. Taken together, these results show that hypothalamic α-MSH immunoreactivity is significantly higher in the C57BL/6J strain relative to 129/SvJ strain of mice.

Immunoreactivity of α-MSH in the MA. Figure 3*D* shows data representing the average densities of α-MSH immunoreactivity in the MA of 129/SvJ and C57BL/6J given

i.p. injection of saline (n = 8/strain), a 1.5 g/kg dose of ethanol (n = 8/strain) or a 3.5 g/kg dose of ethanol (n = 9/strain), and representative photomicrographs of α-MSH immunoreactivity in the MA of 129/SvJ and C57BL/6J mice are presented in Fig. 4*D*,*H*, respectively. As above, a two-way ANOVA performed on these data revealed a significant main effect of mouse strain [$F(1, 44) = 7.85$, $p < 0.0125$], but neither the dose [$F(2, 44) = 1.9$, $p > 0.0125$] nor interaction [$F(2, 44) = 0.2$, $p > 0.0125$] effects achieved statistical significance. In contrast to the hypothalamic regions examined, α-MSH immunoreactivity in the MA was significantly lower in C57BL/6J mice relative to 129/SvJ mice.

DISCUSSION

The most important observation is the present work is that acute administration of a 3.5 g/kg dose of ethanol caused a significant increase in AgRP immunoreactivity in the ARC of C57BL/6J mice, an effect that was not evident in 129/SvJ mice. Strain differences in AgRP immunoreactivity are not likely secondary to strain differences in ethanol metabolism as C57BL/6J and 129/SvJ mice showed similar BECs 2 hours following injection of a 3.5 g/kg dose of ethanol. Additionally, since there were no strain differences in body weight, absolute dosing volume is also not an explanation for strain differences in ethanol-induced AgRP immunoreactivity. Since AgRP plays a role in the modulation of caloric intake, it might be argued that the extra calories associated with ethanol administration were the cause of increased AgRP immunoreactivity in mice injected with the 3.5 g/kg dose. However, reduced caloric intake stemming from fasting is associated with increased AgRP immunoreactivity in the hypothalamus of rats, mice, and sheep (Dunbar et al., 2005; Fetissov et al., 2005; Wagner et al., 2004). In contrast, increasing caloric load (Chang et al., 2005; Ziotopoulou et al., 2000) and hyperphagia in obese tub/tub mice (Backberg et al., 2004) are associated with decreased AgRP expression. Taken together, these previous findings make it unlikely that increased AgRP immunoreactivity in the present report was caused by the calories inherent in ethanol.

With IHC procedures, increased immunoreactivity in response to ethanol exposure could indicate that ethanol facilitates normal signaling via increased production of AgRP. Alternatively, ethanol-induced increases of AgRP immunoreactivity could reflect blunted AgRP signaling, via an attenuation of release and/or augmentation of AgRP re-uptake into presynaptic terminals. While either ethanol-induced increases or decreases in AgRP signaling are possible, the most significant observation in the present work is that ethanol appears to have direct affects on central AgRP activity. This being said, based on previous observations that have implicated AgRP signaling in the modulation of ethanol consumption, we suggest that ethanol-induced increases of AgRP immunoreactivity likely reflect an increase of AgRP signaling. First, central infusion of the AgRP-(83 to 132) fragment increases (Navarro et al., 2005), while genetic deletion of AgRP reduces (Navarro et al., 2009), ethanol self-administration by C57BL/6J mice. Second, C57BL/6J mice show twice the level of ethanol drinking relative to 129/SvJ mice when offered a 10% ethanol solution (Belknap et al., 1993) and here we found that only C57BL/6J mice showed ethanol-induced increases of AgRP. While it is not completely clear if ethanol drinking promotes increases of AgRP immunoreactivity in C57BL/6J mice or if ethanol-induced AgRP immunoreactivity contributes to the high level of ethanol drinking characteristic of this strain, an interesting possibility is that ethanol-induced increase of AgRP signaling is part of mechanism that involves a positive feedback loop, such that ethanol intake stimulates AgRP which in turn promotes further excessive binge-like drinking. In fact, AgRP immunoreactivity has been identified in brain regions implicated in ethanol consumption, including the VTA, NAc, amygdala, bed nucleus of the stria terminalis, and lateral septum (Bagnol et al., 1999), and we have recently shown that AgRP positively modulates binge-like ethanol drinking in C57BL/6J mice (Navarro et al., 2009). Such a positive feedback loop has also been proposed for the neuropeptide galanin. As with AgRP, ethanol stimulates hypothalamic galanin activity and central galanin administration promotes ethanol consumption (Lewis et al., 2005; Schneider et al., 2007). It will be important in future work to determine if C57BL/6J mice show increased AgRP immunoreactivity following a bout of binge-like ethanol drinking. It should be noted that because C57BL/6J and 129/SvJ mice have also been reported to show differences in other ethanol-related phenotypes, such as ethanol-induced sedation (Homanics et al., 1999), AgRP signaling may be involved with any number of neurobiological responses to ethanol that differ between these strains.

The second observation of interest in the present report is that there were region-specific differences in α-MSH immunoreactivity between strains with C57BL/6J mice showing significantly higher α-MSH immunoreactivity in regions of the hypothalamus and lower α-MSH immunoreactivity in the MA relative to 129/SvJ mice. Additionally, the significant main effect of mouse strain in AgRP immunoreactivity indicates that C57BL/6J mice also display a general increase of AgRP in the ARC when compared with 129/SvJ mice. While α-MSH and AgRP have been implicated in food intake and body weight regulation (Sainsbury et al., 2002), the present observations that C57BL/6J and 129/SvJ mice showed statistically similar food intake and body weight suggests that strain differences in α-MSH and AgRP immunoreactivity are not likely related to strain differences in feeding and energy homeostasis. Because C57BL/6J mice drink on average twice as much 10% ethanol as 129/SvJ mice (Belknap et al., 1993) and are less sensitive to the sedative/hypnotic effects of ethanol (Homanics et al., 1999), and in light of the gowning evidence suggesting a role for α-MSH and AgRP ethanol-related phenotypes (Kokare et al., 2008; Navarro et al., 2003, 2005, 2008, 2009; Ploj et al., 2002), it is tempting to speculate that the strain differences in α-MSH and AgRP immunoreactivity contribute to the differences in neurobiological responses to ethanol that are characteristically observed between

C57BL/6J and 129/SvJ mice. However, given that these peptides modulate a diverse set of neurobiological functions, additional work is needed to determine if strain differences in α-MSH and AgRP contribute to C57BL/6J and 129/SvJ strain differences in neurobiological responses to ethanol.

Previous research has shown that chronic exposure to ethanol significantly reduced (Navarro et al., 2008), while abstinence following chronic ethanol exposure increased (Kokare et al., 2008) endogenous α-MSH immunoreactivity in specific brain regions of Sprague-Dawley rats. The absence of an effect of acute ethanol administration on α-MSH immunoreactivity in the present work may suggest that ethanol-induced changes in α-MSH immunoreactivity gradually emerge with chronic exposure. However, an acute i.p. injection of a 2.0 g/kg dose of ethanol was reported to decrease α-MSH immunoreactivity in regions of the hypothalamus and amygdala of rats (Kokare et al., 2008), raising the possibility that there are species (mice vs. rats) differences in α-MSH immunoreactivity in response to acute ethanol injections. However, it should be noted that in the rat study (Kokare et al., 2008), animals injected with ethanol were compared with a group of rats that had consumed a nutritionally balanced liquid diet (in place of food) for 15 days, which raises the possibility that group differences in α-MSH immunoreactivity were related to factors other than acute ethanol exposure, including differences in caloric intake and a host of other procedural differences. Nonetheless, before ruling out a role for endogenous α-MSH in the modulation ethanol's acute effects, further characterization is required (e.g., in other strains or species, with additional doses of ethanol, following ethanol self-administration, and with other techniques for assessing protein levels such as Western blotting).

One caveat that must be considered is that stress associated with i.p. injection of ethanol produced nonspecific effects on α-MSH and/or AgRP immunoreactivity. In fact, stress exposure increases α-MSH release into the blood-stream (Goudreau et al., 1993; Liu et al., 2007) and blunts central AgRP signaling (Kas et al., 2005). While we did not observe ethanol-induced alterations of α-MSH immunoreactivity, possible stress-induced increases of α-MSH immunoreactivity may have masked the attenuating effects of ethanol on α-MSH (Navarro et al., 2008). However, it is unlikely that the stress associated with ethanol injections accounts for the strain differences observed with α-MSH immunoreactivity data as there were no significant dose by strain interaction effects (that is, strain differences were evident regardless of saline or ethanol treatment). Furthermore, since foot shock-induced stress decreases AgRP mRNA in the ARC which is associated with evidence of blunted AgRP release (Kas et al., 2005), ethanol-induced increases of AgRP immunoreactivity in the ARC are likely not related to stress. Finally, while the effects of a ketamine/xylazine (K/X) anesthetic on α-MSH or AgRP immunoreactivity have not been assessed, previous work has revealed that rats anesthetized with K/X failed to show increased c-Fos immunoreactivity in all but 1 brain region examined relative to awake rats when brains were collected 2 hours after drug administration. Halothane anesthesia, on the other hand, induced elevations of c-Fos immunoreactivity in all the 7 brain regions that were examined (Roda et al., 2004). In light of these observations with c-Fos immunoreactivity, we believe that it is unlikely that K/X administration influenced α-MSH or AgRP immunoreactivity in the present report.

In conclusion, the present report provides novel evidence that acute exposure to ethanol induces a dose-dependent increase in AgRP immunoreactivity in the ARC of ethanol preferring C57BL/6J mice, but not in moderate ethanol drinking 129/SvJ mice. This observation suggests that endogenous AgRP signaling may contribute to some of the differences in neurobiological responses to ethanol between these strains, including ethanol consumption and/or ethanol-induced sedation. It will be important in future studies to determine if excessive binge-like ethanol consumption in C57BL/6J mice also promotes increases of AgRP immunoreactivity. Finally, while previous work shows that changes of endogenous α-MSH immunoreactivity are present following chronic ethanol exposure and ethanol withdrawal, the current observations failed to show changes in α-MSH immunoreactivity following acute ethanol administration. Taken together, an interesting possibility is that changes in α-MSH signaling over the course of long-term ethanol exposure contributes to ethanol dependence, whereas increases of AgRP signaling in response to acute ethanol administration contributes to the immediate neurobiological responses to ethanol, including those that modulate the reinforcing properties of ethanol and binge-like drinking. Thus, compounds which target MCRs may prove to have therapeutic value in the treatment excessive ethanol consumption and/or the symptoms associated with ethanol dependence and withdrawal.

ACKNOWLEDGMENTS

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1017

A LOW DOSE OF THE MELANOCORTIN AGONIST MTII SYNERGISTICALLY AUGMENTS NALTREXONE-INDUCED ATTENUATION OF BINGE-LIKE ETHANOL DRINKING IN C57BL/6J MICE

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The central polypeptide precursor proopiomelanocortin (POMC) gives rise to beta-endorphin, an endogenous opioid peptide, and the melanocortin (MC) peptides including alpha-melanocyte stimulating hormone. Opioid receptor antagonists, such as naltrexone (NAL), have been demonstrated to reduce ethanol consumption in rodents, and a growing body of evidence indicates that MC receptor agonists blunt ethanol intake. Interestingly, central opioid and MC pathways have been demonstrated to interact in their modulation of nociception and feeding behavior. Since opioids and MC peptides modulate ethanol consumption, the goal of the present work was to determine if these peptides, when presented in combination, interact additively or synergistically in the modulation of binge-like ethanol drinking in C57BL/6J mice. We used drinking in the dark procedures, an established model of binge-like ethanol drinking, to first established dose-response effects of intraperitoneally (i.p.) injected NAL or the MC agonist MTII (0, 0.3, 3.0, and 10 mg/kg for each drug) on binge-like drinking. Based on these data, we established the ED_{20} and ED_{30} for each drug, and then combined the low (ED_{20}) and high (ED_{30}) dose of each drug with the dose-response range of the other drug. Results showed that MTII was 3.4-fold more potent than NAL in blunting binge-like ethanol drinking (based on ED_{50} values). MTII was also more effective, as the 10 mg/kg dose of MTII produced a 72% reduction of binge-like ethanol drinking while this same dose of NAL reduced drinking by only 49%. When administered in combination, the low ED_{20} (but not the ED_{30}) dose of MTII (0.26 mg/kg) shifted the NAL dose-response curve to the left by a factor of 7 (i.e., NAL was 7-fold more potent when administered in combination with MTII relative to when it was administered alone). Subsequent isobolographic analyses of these data showed that MTII synergistically augmented the ability of NAL to blunt binge-like ethanol drinking. NAL shifted the MTII dose effect curve to the left, but this effect was additive. The present results show that a low dose of MTII synergistically potentiates the ability of NAL to blunt binge-like ethanol drinking. These observations suggest that MC receptor agonists may improve the therapeutic effectiveness of NAL in the treatment of alcohol abuse disorders when these drugs are given in combination. (Supported by NIH grants AA013573 and AA015148, and the Department of Defense grant W81XWH-09-1-0293).

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THE C-TERMINAL FRAGMENT OF THE CORTICOTROPHIN RELEASING FACTOR BINDING PROTEIN (CRF-BP) POSITIVELY MODULATES CRF-RECEPTOR 2 ACTIVATION

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A stress response is believed to involve the corticotropin releasing factor (CRF) system. Deregulation of the CRF system at any point can lead to a variety of psychiatric disorders, such as anxiety, depression, post-traumatic stress disorder, and alcohol use disorder. The precise role of corticotrophin releasing factor binding protein (CRF-BP) in the brain is still the subject of intense investigation. In the periphery, it is believed CRF-BP, due to the higher affinity with its endogenous ligand, plays a buffer role by reducing the amount of free CRF. In the CNS however, the interaction of CRF-BP with CRF-receptor 2 (CRF-R2) results in increased of receptor responsiveness to CRF. CRF-BP is susceptible to autocatalytic proteolysis yielding a larger N-terminal fragment of 27-kilodalton, CRF-BP(27kD), which retains the binding site for CRF and a smaller, 9.6-kilodalton C-terminal fragment, CRF-BP(10kD) with no apparent physiological or pathological role. Loss of the CRF-BP gene in mice has been associated with increased anxiety-like behavior.

Using a novel cellular-based assay, we have shown here, that CRF-BP(10kD) is responsible for enhancing CRF-induced CRF-R2 signaling. Additionally, CRFBP -/- mice exhibited an increase in anxiety-like behavior as compared to their CRFBP +/+ littermates in the open field and elevated plus maze tests.

Our findings support the hypothesis that CRF-BP in a target for addiction and specifically CRF-BP(10kD) may act as endogenous allosteric modulator of CRF-R2 signaling. Our long term goal is to identify small molecules and novel ligands that target this CRF-BP(10kD) interaction with CRF-R2 to develop pharmacotherapies used for stress and to treat alcohol use disorders (AUDs).

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ALCOHOL EFFECTS ON SERUM OXYTOCIN LEVEL IN ALCOHOLICS

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Oxytocin (OXT), a 9-amino acid peptide produced by the posterior pituitary gland and other brain areas, plays a key role in facilitating social behaviors, including maternal behavior and pair-bonding. Oxytocin also possesses anxiolytic, stress reducing and anticraving effects, which suggests that OXT could play a role in the neurobiology of alcohol dependence. Acute alcohol is a potent inhibitor of OXT release and chronic alcohol exposure has neurotoxic effects on OXT neurons and induces behavioral deficits suggestive of a central OXT deficiency. However, there are few studies that administer OXT to animals or alcohol dependent humans to determine its effect on drinking. We recently conducted a experiment to measure OXT in actively drinking alcohol dependent humans. OXT was measured using a multiplexed, competitive format immune-assay (Millipore, Billerica, MA) for the detection of neuropeptides in serum using a Bioplex analyzer (BioRad, Hercules, CA). Non-treatment seeking alcohol dependent subjects recruited for a laboratory alcohol self-administration study (n=34) had elevated plasma OXT (420± 29 pg/ml) compared to non-alcoholic, social drinker controls (211± 12 pg/ml). Our data confirm the increased OXT levels previously observed by Marchesi et al (1997). Nine of the alcohol dependent subjects subsequently participated in an alcohol self-administration experiment. Baseline (recently drinking, but not intoxicated) OTX levels (352±57 pg/ml) were elevated compared to the non-alcoholic controls. Six weeks later, pre-drinking OXT levels were still increased at 523±68 pg/ml (n=7). These subjects then consumed one or more alcohol drinks in an alcohol self-administration experiment and blood OXT levels were repeated 1 hour later. Post-drinking blood OXT levels were decreased during intoxication (238± 47 pg/ml), although the pre-post difference was not significant, probably due to different amounts of alcohol consumed. The data are consistent with an increase in peripheral oxytocin levels in actively drinking alcoholics. Alcohol intoxication still reduces oxytocin levels in these individuals, although the levels remain elevated compared to non-alcoholics. The increased peripheral oxytocin levels are surprising, given the behavioral deficits, increased anxiety and stress seen in alcohol dependence that are more indicative of a central deficiency of OXT. The discrepancy may be due to differences in central and peripheral oxytocin regulation.

1020

IDENTIFICATION OF NOVEL PEPTIDES THAT ALTER ETHANOL MODULATION OF BK CHANNEL FUNCTION

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The BK channel is an evolutionarily-conserved target of ethanol. Genetic manipulation of the BK channel affects behavioral sensitivity to ethanol in a numerous of model systems. Several studies suggest that the BK channel is a viable therapeutic target for alcohol intoxication and addiction. To achieve this end, we have developed a screen identifying peptides that alter BK channel function. Using a monovalent phagemid display technique, we screened 30 million 9 amino acid sequences for those that bind to the BKα channel. Twenty-seven unique peptides remained after panning for sequences that bind to the human BKα channel but not the rat SK2 channel or human glycine α1 receptor. Sequences bearing clusters of positively charged amino acids were enriched 100-300 fold. Three motifs were enriched 3000–6000 fold. To rapidly screen for functional effects of select peptides, we made use of the nematode, *C. elegans*. Peptides were tested for their abilities to alter locomotion, an ethanol- and BK-channel dependent behavior in *C. elegans*. Locomotion is suppressed by enhancing BK channel activity, and acute exposure to pharmacologically-relevant concentrations of ethanol enhances BK channel function. A peptide, pskan4, was identified that had no effect on locomotion when administered alone but enhanced ethanol suppression of locomotion ($p<0.0001$). The effect of this peptide was abolished in BK knockouts. These data suggest that pskan4 enhances the ethanol-dependent increase in BK channel activity, but does not alter activity in the absence of ethanol. Another peptide, pskan1, had BK channel- but not ethanol-dependent effects on locomotion. While these two peptides had motifs enriched 4000–6000 fold, a peptide without a highly enriched motif caused only non-specific behavioral effects. Further, a similar 9 amino acid peptide not selected in the panning procedure did not have significant effects on locomotion indicating that the screening technique was specific for the desired target. Overall, these findings show that we have developed and successfully employed a screen for identifying and characterizing novel peptides that alter BK channel-dependent behavior in the presence of pharmacologically-relevant concentrations of ethanol. Future electrophysiological testing will characterize the abilities of peptides to alter BK channel function in the presence or absence of ethanol. This screening technique can be applied to identify peptide modulators of other ion channels.

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NEUROSCIENCE 2012

Presentation Abstract

Program#/Poster#: 454.16/P10

Presentation Title: The melanocortin (MC) receptor agonist, melanotan-ii (MTII), blunts binge-like ethanol drinking in mice via the MC-4 receptor (MC4R)

Location: Hall F-J

Presentation time: Monday, Oct 15, 2012, 4:00 PM - 5:00 PM

Authors: ***J. OLNEY**, G. SPROW, T. THIELE;
Dept. of Psychology, Univ. of North Carolina At Chapel Hill, Chapel Hill, NC

Abstract: Recent data have implicated the MC system in modulating voluntary ethanol consumption. Administration of MTII, a nonselective MC receptor agonist, reduces voluntary ethanol consumption in C57BL/6J mice. Interestingly, central infusion of MTII failed to alter ethanol drinking in mutant mice lacking normal expression of the MC4R (MC4R-/- mice), demonstrating that the effects of central MTII on ethanol drinking involves signaling through the MC4R. Despite the known role of MC4R in voluntary ethanol consumption, the role of the MC4R in binge-like ethanol drinking remains unknown. Therefore, the present study took advantage of "drinking in the dark" procedures, an established animal model of binge-like ethanol drinking, to assess the role of the MC4R in binge-like ethanol intake. During this procedure, mice are given access to ethanol (20% v/v) in place of water beginning 3 hours into the dark cycle (12 hour:12 hour light:dark cycle). On days 1-3 of the DID procedure mice have access to ethanol for 2 hours. The effect of MTII on binge-like ethanol drinking is assessed on the fourth day when access to ethanol is extended to four hours. To assess the role of the MC4R in modulating this pattern of excessive consumption, the effects of MTII on binge-like ethanol drinking were assessed in MC4R-/- and littermate MC4R+/+ mice. Two studies were performed, in which MTII was administered via intracerebroventricular (i.c.v.) infusions (0, 0.25, or 0.50 µg) or peripherally in intraperitoneal (i.p.) injections (0, 2.5, or 5.0 mg/kg) three hours prior to ethanol access on day 4. Consistent with voluntary consumption data, i.c.v. infusion of MTII blunted binge-like ethanol drinking in wild-type MC4R+/+, but not MC4R-/-, mice. Furthermore, as with voluntary consumption procedures, i.p. injection of MTII (5.0 mg/kg) significantly blunted DID ethanol intake in both MC4R-/- and MC4R+/+ mice during the first hour of ethanol drinking. Together, these findings suggest that the MC4R is required for the central actions of MTII on excessive ethanol intake

but may not modulate the peripheral effects of MTII on binge-like ethanol consumption.

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Keyword(s): ETHANOL

PEPTIDE

KNOCKOUT MICE

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NEUROSCIENCE 2012

Presentation Abstract

Program#/Poster#: 454.15/P9

Presentation Title: Melanocortin receptor signaling in the lateral hypothalamus modulates binge-like ethanol consumption in C57BL/6J mice

Location: Hall F-J

Presentation time: Monday, Oct 15, 2012, 3:00 PM - 4:00 PM

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Abstract: The melanocortin (MC) system has previously been implicated in modulating neurobiological responses to ethanol. The goal of the present study was to assess the role of this system, specifically within the lateral hypothalamus, in modulating binge-like ethanol consumption using the “drinking in the dark” (DID) protocol. In this model, 20% (v/v) ethanol is presented to mice in place of water for 2 hr, 3hr into the dark cycle, for 3 days. On day 4, the “binge” day, this access is extended to 4 hr. This protocol, which capitalizes on the innate consummatory patterns of rodents, generates pharmacologically relevant blood ethanol concentrations (BECs) following voluntary ethanol consumption. In this study, male C57BL/6J mice (n=28) were implanted with bilateral cannulae targeting the lateral hypothalamus. Following recovery, the DID protocol was initiated; three hours before ethanol presentation on the “binge” day, animals received a bilateral infusion of the non-selective MC receptor agonist melanotan-II (MT-II; 0.5nmol/0.5µ l/side), the non-selective MC receptor antagonist agouti-related protein (AgRP; 0.1nmol/0.5µ l/side), or a selective MC-4 receptor (MC4R) agonist (1.0nmol/5µl/side). BECs were measured immediately following the final binge-like drinking session. Additionally, all mice underwent a sucrose control study identical to the methods described above, except that 10% (w/v) sucrose was presented in place of the 20% ethanol. Mice that received bilateral infusion of either MT-II or the MC4R agonist showed significant reductions in binge-like ethanol consumption during the final session relative to vehicle-treated animals, with the most robust decrease in consumption apparent in the first hour of the final binge session. Mice that received bilateral infusion of AgRP showed a significant increase in ethanol consumption relative to vehicle-treated animals, with the most robust increase occurring during the first hour of the final binge session. Importantly, neither agonism nor antagonism of the MC system impacted sucrose

consumption in MT-II- or AgRP-treated mice. Together, these results implicate the MC system within the lateral hypothalamus as playing an integral role in specifically modulating binge-like ethanol consumption.

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REPEATED BINGE-LIKE ETHANOL INTAKE IN C57BL/6J MICE LEADS TO DECREASED α-MSH IMMUNOREACTIVITY (IR) AND INCREASED AGRP IN KEY BRAIN REGIONS

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Recent data has shown that the melanocortin (MC) system modulates neurobiological responses to ethanol. MC receptor (MCR) agonists decrease, while antagonists increase, ethanol consumption. Chronic exposure via an EtOH-containing diet has been shown to cause a significant decrease in α-MSH immunoreactivity (IR) in thalamic and amygdalar regions. Additionally, mice lacking the endogenous MCR antagonist agouti-related protein (AgRP) show a decrease in binge-like ethanol consumption. The purpose of this study was to examine α-MSH and AgRP IR following cycles of binge-like EtOH exposure. Eighty male C57BL/6J mice were randomly assigned to one of eight groups: mice received 1, 3, or 6 four-day binge-like drinking sessions with EtOH (20%, v/v) or sucrose (thus: 1-EtOH, 1-Suc, 3-EtOH, 3-Suc, 6-EtOH, and 6-Suc), continuous water (CW) or continuous ethanol (CE). Immediately after bottles were removed following the final drinking session, tail bloods were collected to measure blood ethanol concentrations (BECs). Brains were collected and processed for DAB IR. EtOH consumption in 1-EtOH and 3-EtOH mice was significantly lower than that of CE mice on the final binge day; there were no differences between sucrose groups. Mice in the CE group also achieved significantly lower BECs than mice in either the 3-EtOH or 6-EtOH groups. All EtOH-treated groups showed attenuated α-MSH IR in the lateral hypothalamus (LH) relative to the CW group. The dorsomedial (DMH) and arcuate nuclei (ARC) of the hypothalamus showed treatment-dependent decreases in α-MSH IR. In these three regions, binge-like ethanol drinking-induced attenuation of α-MSH IR was not associated with alterations of α-MSH IR related to sucrose drinking. Ethanol-induced changes were not evident in the nucleus accumbens (NAc), central medial thalamus (CMTH), or central amygdala (CeA). AgRP IR was significantly increased in the ARC in both CE and the 6-EtOH mice. No changes in AgRP IR were detected in the NAc, CMTH, or DMTH. Together, these results suggest that ethanol consumption induces alterations of α-MSH and AgRP, changes that become more robust with greater ethanol exposure. Interestingly, reduction of α-MSH was evident after one binge-like drinking cycle, suggesting that endogenous α-MSH modulates neurobiological responses associated with binge-like ethanol drinking. (Supported by NIH grants AA013573, AA015148 and AA019839 and the Department of Defense grants W81XWH-06-1-0158 and W81XWH-09-1-0293).

0942

BINGE DRINKING IN ADOLESCENCE INCREASES DELTA OPIOID RECEPTOR FUNCTION IN THE DORSAL STRIATUM THAT CONTRIBUTES TO ETHANOL CONSUMPTION IN ADULTHOOD

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Binge drinking during adolescence increases the propensity for alcohol use disorders (AUDs) later in adult life, however, the mechanisms that drive this are incompletely understood. The delta opioid peptide receptor (DOP-R) has been shown to play a role in the reinforcing effects of ethanol and administration of DOP-R ligands to rodents modulates ethanol intake. The objective of this study was to investigate the role and function of the DOP-R with long-term high ethanol intake from adolescence into adulthood. Groups of rats (P28, P56, P154) were given intermittent access to 20% ethanol or water only for different lengths of time (0–6 months). Tail-flick DOP-R-mediated analgesia and [^{35}S]GTPγS binding in rat brains were measured in each group. We show that DOP-R activity in the dorsal striatum and DOP-R-mediated analgesia changes during development, being highest during early adolescence and significantly reduced in middle and late adulthood. We show that intermittent and heavy ethanol intake in peri-adolescent and young adult rats leads to an increase in the function of the DOP-R in late adulthood rats. We show that chronic intermittent ethanol but not continuous ethanol or water consumption increases DOP-R [^{35}S]GTPγS binding specifically in the dorsal striatum. Multiple administrations of the DOP-R antagonist, naltrindole, for 28 days produces selective, long-lasting and permanent reductions of voluntary ethanol consumption such that when naltrindole treatment was terminated, ethanol consumption was maintained at a reduced level for the next 28 days, compared to post–vehicle-treated rats. Rats given multiple administrations of naltrindole for 28 days have attenuated DOP-R function in the dorsal striatum after naltrindole treatment was terminated for a further 28 days, compared to vehicle-pretreated rats. These findings show that long-term high ethanol intake from adolescence increases DOP-R activity into adulthood and that the DOP-R function in the dorsal striatum contributes to the escalation and maintenance of ethanol consumption. These studies suggest the DOP-R plays a significant role in ethanol-mediated behaviors and suggest that targeting the DOP-R is an alternative strategy for the treatment of AUDs. This work was supported by funding from the State of California for Medical Research through UCSF to S.E.B and Department of Defense Grant W81XWH-06-1-0240 to S.E.B. S.E.B. and C.K.N. were supported in part by the NARSAD Young Investigator Award.

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DISTURBANCES IN BEHAVIOR AND ENKEPHALIN GENE EXPRESSION DURING THE ANTICIPATION OF ETHANOL IN HIGH-DRINKING RATS

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The anticipation of ethanol can activate specific brain circuits involved in ethanol-seeking behavior. Although past studies have focused on the dopamine reward system in driving such behaviors, more recent studies point to an important role of the opioid peptide, enkephalin (ENK), in promoting enhanced ethanol drinking behavior by acting within several hypothalamic and mesocorticolimbic regions. The goal of the current study was to determine whether a subpopulation of Sprague-Dawley rats that overconsume ethanol exhibit disturbances in behavior during the anticipation of ethanol and also in the expression of ENK within the paraventricular nucleus of the hypothalamus (PVN) and medial prefrontal cortex (mPFC), regions known to promote consummatory behavior and impulsivity, respectively. To this end, rats were trained for 2 weeks in a limited access (3h/d) paradigm to consume 20% ethanol solution right at dark onset. With ethanol intake being positively correlated from day to day (r = +0.76, $p < 0.01$), two distinct subgroups of animals (n = 8/group) were formed, a low-drinking (LD) group, which consumed an average of 0.6 g/kg/3 h, and a high-drinking group (HD), which consumed considerably more ethanol (1.7 g/kg/3 h) and showed increased blood ethanol content (65±8.9 vs. 21±5.6 mg/dl, $p < 0.05$). In order to measure ethanol-seeking behavior in these animals during the anticipation of the ethanol, locomotor (ambulatory distance) and exploratory (vertical counts) behaviors in an open field activity chamber were measured for 10 minutes just prior to daily ethanol access. The results showed in the HD compared to LD rats a significant increase in ambulatory distance traveled (+43%, $p < 0.05$) and vertical counts (+47%, $p < 0.05$) during the anticipation of ethanol. Next, in order to examine disturbances in ENK expression during this anticipation period, animals were characterized as HD and LD in an identical manner as above and sacrificed just before their scheduled access to ethanol. Using quantitative real-time PCR to measure gene expression, the data showed increased ENK mRNA in the PVN (+46%, $p < 0.05$) and mPFC (+72%, $p < 0.05$) of HD compared to LD rats as they were anticipating their daily bouts of ethanol. Together these data suggest that, in animals prone to drinking excess amounts of ethanol, anticipatory behaviors related to ethanol-seeking may be driven by enhanced ENK expression within the PVN and mPFC, regions known to stimulate consummatory and impulsive behaviors.

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LONG-TERM ETHANOL BINGE DRINKING ALTERS DENDRITIC MORPHOLOGY IN LIMBIC BRAIN REGIONS OF LONG-EVANS RATS

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The early onset of ethanol intake often develops into high levels of episodic binge drinking increasing the likelihood of chronic intake or alcoholism. Evidence suggests that chronic ethanol intake produces changes in the structure of dendrites and spines in the nucleus accumbens of rodents. Other studies have determined that chronic human alcohol drinkers have cortical neuronal alterations in the terminal branches of dendrites, in opposing directions on proximal versus distal branches. Our objective was to determine whether moderate long term binge drinking produces alterations in dendritic branching and complexity in the nucleus accumbens, a brain region involved in motivational aspects of ethanol self-administration. Male Long-Evans rats self-administered 2% sucrose/10% ethanol (2S10E) or 2% sucrose (2S) by performing a daily fixed number of lever presses (response requirement, RR 20) for one year. Mean ethanol intake was 1.3 g/kg/day and was consumed in a daily 20 minute session. At the end of the study fresh brains were collected and impregnated using the rapid Golgi-Cox staining method, and sectioned on a cryostat microtome. Dendritic morphological analysis was performed on medium spiny neurons in the nucleus accumbens using stereology. The results indicate that there were significant increases in the 2S10E versus 2S groups: 40% in the number of branch points ($p < 0.001$); 45% in the total number of primary branches ($p < 0.01$); 38% in the total number of branch tips ($p < 0.001$); 30% in the mean number of terminals, for only second order branches ($p < 0.01$). The total dendritic arbor and average segment length were not significantly altered. These results indicate that a moderate dose of long term drinking of 2S10E versus 2S produced an increase in dendritic branching possibly due to the lack of pruning or the result of a beneficial environmental interaction while drinking ethanol. The results in this study are consistent with evidence that the number of second and third order terminal branches increases in human chronic ethanol drinkers. There is evidence that ethanol related neuronal damage is regional specific. The current study is the first to demonstrate that repeated moderate ethanol binge drinking in rodents beginning in adolescence and continuing through adulthood for over a year alters dendritic branching in a brain region associated with motivational aspects of alcohol drinking. This work was supported by NIAAA grant K01 AA015194.

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GHRELIN SYSTEM IN ALCOHOL-DEPENDENT SUBJECTS: ROLE OF BASELINE PLASMA GHRELIN LEVELS IN ALCOHOL CRAVING AND INTAKE

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Ghrelin is a gut-brain peptide acting as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R). Preclinical studies demonstrate that ghrelin activates the cholinergic-dopaminergic reward link, and the central ghrelin action is required for stimulation of the reward system by alcohol. Human studies show that alcohol acutely suppresses blood ghrelin levels in both healthy controls and alcoholics. Moreover, a significant positive correlation between plasma ghrelin levels and the Obsessive-Compulsive Drinking Scale (OCDS) craving score was described in actively drinking alcoholics. The present study was a 12-week longitudinal study with 42 alcohol-dependent individuals. The goal of this study was to perform repeated ghrelin determinations, as well as to investigate the relationship between ghrelin and alcohol craving and intake. Hormone determinations (ghrelin, GH) and craving (OCDS, PACS) measurements were performed at baseline (T0), and then at 2 weeks (T1), 6 weeks (T2) and 12 weeks (T3). There were no statistical changes on ghrelin levels from T1 to T3, when the total group of alcoholics was considered. However, when abstinent alcoholics at T3 and non-abstinent alcoholics at T3 were analyzed separately, we found that baseline ghrelin levels (T0) were higher in the non-abstinent alcoholics and decreased during the 12 weeks, while baseline ghrelin levels (T0) were lower in the abstinent alcoholics and increased during the 12 weeks. There was a significant difference for ghrelin levels between non-abstinent vs. abstinent alcoholics ($p = 0.012$). Moreover, baseline ghrelin levels were significantly and positively correlated with the PACS craving score at T1 ($p = 0.012$), and with both PACS and OCDS craving scores at T2 and T3. Ghrelin did not correlate with GH levels. The present study shows significant differences in the changes of ghrelin levels between non-abstinent alcoholics and abstinent alcoholics. Ghrelin also correlated significantly with craving measurements. Together, these results might suggest that higher ghrelin levels reflect a higher risk of alcohol drinking and relapse. Consistent with previous animal and human studies, this study leads to hypothesize that the ghrelin system represents a new pharmacological target for the treatment of alcohol dependence.

018

ACUTE ETHANOL EXPOSURE DIFFERENTIALLY AFFECTS OREXIN NEURONS OF THE PERIFORNICAL VERSUS LATERAL HYPOTHALAMUS

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Our previous studies have examined the effects of ethanol on hypothalamic peptide systems and identified a positive feedback loop, in which peptides of the paraventricular nucleus such as enkephalin and galanin stimulate ethanol intake, while ethanol in turn stimulates the expression of these same peptides. Recently, orexin (OX), a peptide produced mainly by cells in the perifornical lateral hypothalamus (PFLH), has been shown to play an important role in mediating the rewarding aspects of ethanol consumption. However, there is little evidence showing the effects that ethanol itself may have on the OX peptide system. In order to understand the feedback relationship between ethanol and the OX system, the current investigation was designed to measure OX expression in the PFLH following acute ethanol intake. The first two experiments tested the impact of acute, oral gavage of a low (0.75 g/kg) or high (2.5 g/kg) dose of ethanol on OX mRNA levels in the PFLH, using real-time quantitative PCR as well as radiolabeled in situ hybridization (ISH). The third experiment used digoxigenin-labeled ISH and immunofluorescence histochemistry to identify more precisely the location of OX neurons in the perifornical (PF) or lateral hypothalamic (LH) regions. The results showed that, compared to water, acute oral ethanol at the lower but not higher dose significantly enhanced OX expression in the PFLH 2 hours after ethanol administration. Additional analyses of OX cells in the PF versus LH regions identified the latter as the primary site of ethanol's stimulatory effect. In the LH but not the PF, acute ethanol increased the density of OX-expressing neurons with 0.75 g/kg ethanol and stimulated OX immunoreactivity with 2.5 g/kg ethanol. These dose-related changes in gene expression were associated with a change in blood ethanol concentration (BEC). Following a rapid increase in levels 15 minutes after oral gavage of both doses, BEC returned to baseline 2 hours after the 0.75 g/kg dose but remained elevated after the 2.5 g/kg dose. Together, these results suggest that acute low dose ethanol, with a small increase in BEC, stimulates OX mRNA expression most potently in the LH, where OX may trigger central mechanisms related to reward. In contrast, the higher dose of ethanol, resulting in a sustained BEC elevation, appears to trigger inhibitory signals, which negatively feed back to reverse this stimulatory effect on OX expression.

019

NEUROPEPTIDE Y (NPY) AND BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF) RESPONSIVITY AFTER ACUTE STRESS IN ALCOHOL-DEPENDENT AND CONTROL SUBJECTS

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NPY and BDNF serve protective and neuroadaptive functions within the central nervous system and modulatory functions in the peripheral system. As such, both NPY and BDNF have been associated with physiological challenges such as stress and substance abuse. Previous studies have found correlations between increased NPY and BDNF expression and alcohol dependence, as well as with chronic and episodic stress. In this study, we examined the effect of acute stress upon both peptides in alcohol-dependent and healthy control populations.
Methods: Four to 6-week abstinent male alcohol-dependent patients were recruited from residential treatment programs and race- and age-matched controls were recruited from the general public. The Trier Social Stress Test (TSST), a public speaking task, was administered at 7 PM. Serum basal samples were obtained 15 and 5 minutes prior to the stressor. Two additional serum samples were collected 5 and 15 minutes after the stressor. Basal measures were averaged and peptide response was determined by the net maximal response following the stressor (â peak). NPY was obtained in 9 controls and 11 patients; BDNF in 7 controls and 10 patients.
Results: There were no significant basal group differences in NPY. â peak increases in NPY were observed in both groups (controls: $p < 0.10$, patients: $p = 0.11$). â peak were not significantly different between groups. A basal group difference was observed in BDNF ($p < 0.09$). â peak increases in BDNF were observed in both groups (controls: $p = 0.11$, patients: $p = 0.16$). â peak were not significantly different between groups.
Conclusion: Non-statistically significant increases in both serum NPY and BDNF followed a behavioral stressor. Group trend differences were limited to higher basal BDNF levels in patients. These findings were limited by small group sizes and marked intra- and inter-subject NPY and BDNF variability. Despite recent headway in elucidating the roles of these two peptides in stress and alcoholism, the high variability in these human populations suggests a complex interplay between NPY, BDNF, and other unknown factors that must be investigated further.
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020

CENTRAL, BUT NOT PERIPHERAL, ADMINISTRATION OF MELANOCORTIN (MC) RECEPTOR AGONISTS REQUIRE THE MC-4 RECEPTOR TO REDUCE ETHANOL INTAKE

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The melanocortin (MC) system is composed of peptides that are cleaved from the polypeptide precursor proopiomelanocortin (POMC). Evidence shows that MC receptor (MCR) signaling modulates ethanol consumption and that administration of ethanol alters central MC peptide immunoreactivity. Previously we found that administration of the potent non-selective MCR agonist malanotan-II (MTII) was similarly effective at reducing ethanol intake in both wild-type mice and in mutant mice lacking the MC-3 receptor. Here, we assessed the contribution of the MC-4 receptor (MC4R) with MC4R-/- and MC4R+/+ mice maintained on a C57BL/6J genetic background. In three separate studies, mice were given intracerebroventricular (i.c.v.) infusion of the selective MC4R agonist cyclo (NH-CH_2- CH_2-CO-His-D-Phe-Arg-Trp-Glu)-NH_2 (0, 1, 3, or 6 μg/1 μl) or MTII (0, 0.5, or 1.0 μg/1 μl), or given intraperitoneal (i.p.) injection of MTII (0 or 5 mg/kg). Results showed that i.c.v. infusion of MTII or the MC4R agonist significantly reduced ethanol drinking in MC4R+/+ mice but failed to influence ethanol intake in MC4R-/- mice. On the other hand, when administered in an i.p. injection, MTII significantly reduced ethanol drinking in both MC4R-/- and MC4R+/+ mice. Similar results were obtained with food intake data. These results show that centrally administered MCR agonists reduce ethanol intake in C57BL/6J mice by signaling through the MC4R. On the other hand, peripherally administered MTII does not require the MC4R in the modulation of ethanol drinking (Supported by NIH grants AA013573 and AA015148 and the Department of Defense grants W81XWH-06-1-0158 and W81XWH-09-1-0293).